RINAMARA MARTINS ROSA

IMPACTOS METABÓLICOS EM *Chlamydomonas reinhardtii* CC503 CULTIVADA COM UREIA EM CONDIÇÕES AUTOTRÓFICAS E MIXOTRÓFICAS

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Botânica, para a obtenção do título de *Doctor Scientiae*.

Orientador: Adriano Nunes Nesi

Coorientador: Marcelo Gomes M. Vieira Vaz

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Assentimento:

Rinamara Martins Rosa

Autora

Adriano Nunes Nesi

Orientador

Dedico este trabalho aos meus pais, Carmem e Mário, e ao meu sobrinho Pedro Henrique. Minha base e motivação diária.

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RESUMO

ROSA, Rinamara Martins, D.Sc., Universidade Federal de Viçosa, maio de 2020. **Impactos metabólicos em *Chlamydomonas reinhardtii* CC503 cultivada com ureia em condições autotróficas e mixotróficas.** Orientador: Adriano Nunes Nesi. Coorientador: Marcelo Gomes Marçal Vieira Vaz.

A ureia por ser uma molécula que contém C e N, e de abundante disponibilidade em ambientes aquáticos é amplamente utilizada como fonte de N. Entretanto, as vias metabólicas nas quais a ureia está envolvida, bem como a regulação das mesmas ainda não são bem conhecidas em microalgas. Assim, o objetivo desse trabalho foi investigar como a cepa de *C. reinhardtii* CC503 responde à utilização de ureia como fonte de N sob condições de autotrofia e mixotrofia por meio de análises fisiológicas e metabólicas. Os resultados obtidos comprovam que o cultivo com ureia configura uma condição de mixotrofia, porém a incorporação do C proveniente da molécula é feita via ciclo de Calvin como a assimilação inorgânica. Ao cultivarmos com acetato e ureia, observou-se que a condição de estresse ocasionada pela metabolização do acetato é atenuada, provavelmente pelo suprimento adicional de C inorgânico da ureia. Adicionalmente, verificou-se os o suprimento de C é um fator impactante na variação temporal dos parâmetros bioquímicos e que o C proveniente da ureia é uma fonte importante desse nutriente principalmente na ausência de acetato. Nossos resultados indicam que a alta razão C:N na presença de acetato torne menos evidentes os efeitos do suprimento de C pela ureia e que a presença dessa fonte orgânica de nitrogênio é capaz de atenuar os efeitos oxidativos do metabolismo de acetato, como inibição da fotossíntese, drástico aumento da velocidade de crescimento e o padrão de acúmulo de compostos.

Palavras-chave: Autotrofia. Carbono. Metabolismo. Mixotrofia. Nitrogênio. Ureia.

ABSTRACT

ROSA, Rinamara Martins, D.Sc. Universidade Federal de Viçosa, May, 2020. **Metabolic responses in *Chlamydomonas reinhardtii* CC503 cultivated with urea in autotrophic and mixotrophic conditions.** Adviser: Adriano Nunes Nesi. Co-adviser: Marcelo Gomes Marçal Vieira Vaz.

Urea, being a molecule that contains C and N, and of abundant availability in aquatic environments is widely used as a source of N. However, the metabolic pathways in which urea is involved, as well as their regulation are still not well known in microalgae. Thus, the objective of this work was to investigate how the strain of *C. reinhardtii* CC503 responds to the use of urea as a source of N under conditions of autotrophy and mixotrophy through physiological and metabolic analyzes. The results obtained prove that the cultivation with urea constitutes a condition of mixotrophy, however the incorporation of C from the molecule is done via the Calvin-cycle as inorganic assimilation. When cultivating with acetate and urea, it was observed that the stress condition caused by the acetate metabolism is attenuated, probably by the additional supply of inorganic C from urea. Additionally, it was found that the supply of C is an impacting factor in the temporal variation of biochemical parameters and that the C from urea is an important source of this nutrient, especially in the absence of acetate. Our results indicate that the high C: N ratio in the presence of acetate makes the effects of urea C supply less evident and that the presence of this organic nitrogen source is able to attenuate the oxidative effects of acetate metabolism, as an inhibition of photosynthesis, drastic increase in the speed of growth and the pattern of accumulation of compounds.

Keywords: Autotrophy. Carbon. Metabolism. Mixotrophy. Nitrogen. Urea.

SUMÁRIO

INTRODUÇÃO GERAL

Microalgas têm sido amplamente estudadas, principalmente por seu potencial biotecnológico para produção de biomassa e de metabólitos de interesse comercial [1–4] Algumas subdivisões de microalgas, sobretudo do filo Chlorophyta, são frequentemente empregadas como modelo para estudos metabólicos e fisiológicos de organismos fotoautótrofos [5–8]. Além da importância biotecnológica estes organismos são bastante estudados com objetivos comparativos, análises moleculares identificaram o grupo como sendo associado à origem dos eucariotos fotoautotróficos e, portanto, a ligação evolutiva entre plantas vasculares e os animais [6,7,9,10].

Nesse contexto, Chlamydomonas reinhardtii é o organismo tido como modelo em boa parte dos estudos realizados até o momento [8,11]. Esta espécie é uma clorófita unicelular, que apresenta um par de flagelos anteriores os quais lhe atribuem motilidade. Esta microalga se reproduz assexuadamente em condições específicas, porém, apresenta também ciclo sexuado que é induzido sob estresse nutricional, principalmente estresse causado pela deficiência de nitrogênio (N) [12,13]. Além das características intrínsecas do organismo, C. reinhardtii possui genoma sequenciado e bem anotado, com técnicas bem consolidadas de transformação e facilidade de manipulação genética o que a torna um organismo atraente para estudos relacionados à fotossíntese, biologia de cloroplastos, assimilação e uso de nutrientes sob diferentes condições de cultivo, além de aspectos que envolvem sua estrutura e morfologia [7,12–18]. Adicionalmente a essas características, Chlamydomonas é capaz de crescer em condições de autotrofia, mixotrofia ou heterotrofia, utilizando neste último caso fontes orgânicas de carbono como o acetato [19,20].

O interesse biotecnológico pela Chlamydomonas e também por outras espécies de microalgas no geral [3], no entanto, tem se sobressaído em relação a outras áreas de

estudo, principalmente em função do potencial comercial desses microrganismos para a produção lipídeos e carboidratos conversíveis a biocombustíveis e proteínas com importância alimentar e farmacêutica [3,21–23]. Neste sentido, a maioria das pesquisas tem focado na manipulação de condições de cultivo e aplicação de estresses nutricionais, como de ferro, N e enxofre, visando o aumento da produtividade de biomassa e de compostos de interesse, como lipídeos e carboidratos [24,25].

O primeiro aspecto explorado com o objetivo de modulação do metabolismo e crescimento são as diferentes condições nutricionais nas quais microalgas são capazes de se desenvolver. As variações do metabolismo energético nesses organismos são determinadas pelas caraterísticas ambientais, como disponibilidade de nutrientes e de luz [6]. Condições autotróficas (fotoautotrofia, no caso de microalgas), consistem no mecanismo de produção de compostos orgânicos complexos utilizando-se recursos químicos como a luz, H2O e compostos inorgânicos para a realização do processo e produção de energia química. A condição de heterotrofia, por sua vez, é caracterizada pela assimilação de compostos orgânicos diretamente do meio sendo estes posteriormente metabolizados e convertidos em energia. Essa condição ocorre para microalgas na ausência de luz e presença de compostos orgânicos como acetato e outras moléculas orgânicas de dois carbonos. Adicionalmente à estas condições as microalgas também apresentam a capacidade de se desenvolver em condição de mixotrofia, que consiste na capacidade do organismo de fixar compostos inorgânicos na produção de energia química simultaneamente à absorção e uso de compostos orgânicos provenientes do meio. No metabolismo mixotrófico temos componentes da autotrofia e heterotrofia atuando conjuntamente no metabolismo da célula, compartilhando produtos e substratos [6,26].

Em C. reinhardtii a principal fonte de suplementação de C onde se estudam os mecanismos de heterotrofia e mixotrofia é o acetato [27]. Este composto orgânico de dois carbonos é facilmente metabolizado, sendo preferencialmente assimilado via enzima

acetil-CoA sintase. Sua utilização, apesar de provocar incremento na produtividade de biomassa, tende a inibir a atividade fotossintética quando comparado às condições de fotoautotrofia. Trabalhos anteriores que objetivaram compreender o fluxo metabólico do acetato sugerem que sua assimilação altere a via das pentoses fosfato e aumente a atividade respiratória da mitocôndria, o que pode se relacionar ao fato de sua metabolização produzir coenzimas reduzidas capazes de alterar o equilíbrio redox na organela. Na presença de luz, o incremento de carbono orgânico fornecido pelo acetato alimenta um ciclo TCA modificado que ignora as etapas de evolução de CO2, em geral atenuando a fotossíntese e estimulando as vias de reserva de carbono, o que é bastante explorado em estudos biotecnológicos [26,27].

Adicionalmente à variação das condições nutricionais, o N é um dos principais nutrientes cujas respostas em microalgas tem sido constantemente investigada, em geral por sua relação direta com o metabolismo do carbono (C) [28–31]. Estudos envolvendo privação de N, demonstram reduções nos níveis de aminoácidos, na biossíntese de ribossomos, na quantidade das enzimas do ciclo do glioxilato [32,33], da via das pentoses fosfatadas e as do Ciclo de Calvin-Benson [14,34]. Em contrapartida, observa-se que a biossíntese de ácidos graxos e as proteínas relacionadas à assimilação de N, ao ciclo dos ácidos tricarboxílicos (Ciclo TCA) e à fosforilação oxidativa aumentaram durante o estresse causado pela deficiência de N [28,35,36]. Em geral, esses estudos embasam a correlação entre privação de N e acúmulo de reservas em microalgas, no entanto, além da concentração de N a fonte através do qual esse elemento é fornecido também é importante.

As algas clorófitas são capazes de assimilar N proveniente tanto em formas orgânicas como purinas, ureia, ureato e aminoácidos, quanto inorgânica como nitrato, nitrito e amônio [33]. Verifica-se que fonte de N fornecida em cultivos está relacionada a alterações que ocorrem no metabolismo de reservas e acúmulo de lipídeos [37,38].

Trabalhos com variação de fontes e privação de N em clorófitas demonstraram que esta condição ocasiona mudanças no padrão de transcritos e na regulação do metabolismo estimulando o direcionamento do carbono da fotossíntese para o ciclo do glioxilato e a gliconeogênese [32,39,40]. A variação no padrão de acúmulo e degradação de compostos também responde à suplementação de carbono, indicando que direcionamento de reservas nas diferentes condições nutricionais possa ocorrer em resposta aos impactos na razão C:N [41–46]. Sabe-se que a razão C:N em microalgas deve estar acima de 5:1, e que em condições de mixotrofia com suplementação por acetato essa razão é praticamente triplicada (14:1), o que pode associar o incremento do crescimento ao aumento da razão C:N [26,47].

As fontes de N inorgânicas comumente utilizadas por clorófitas, dada sua disponibilidade, são amônio (NH_4^+), nitrato (NO_3^-) e nitrito (NO_2^-) [33]. Diversos estudos relacionam o acúmulo de lipídeos a nitrato e nitrito e a redução a amônio [48]. Por sua vez, as fontes orgânicas de N mais comuns são purinas, ureia e aminoácidos [49], sendo dentre essas ureia a fonte geralmente mais estudada como suplementação em cultivos [32]. Sabe-se que este composto orgânico, altamente solúvel, está presente na excreção de animais e representa ao menos 50% do N total utilizado por comunidades fitoplanctônicas oceânicas e costeiras [30,42,50]. Em estudos com produtividade de biomassa concluiu-se que o uso da ureia permite um maior crescimento e acúmulo de lipídeos, comparada ao amônio [29,51]. De forma complementar, o resultado desses estudos com variação em fontes e concentrações de N indicam também a relevância da disponibilidade de C e da relação C:N nos cultivos para o acúmulo de compostos de reserva [30,33,52,53].

A partir dos estudos do genoma de C. reinhardtii, tornou-se possível identificar os componentes responsáveis pelo transporte e assimilação de fontes nitrogenadas nessa alga [18,33]. No caso das fontes inorgânicas de N o transporte ocorre passivamente por proteínas específicas, que se encontram ancorados à membrana plasmática e na

membrana do cloroplasto [33]. Há um grupo de genes que codifica transportadores específicos para cada uma das fontes: AMT1, família de transportadores de amônio; NRT 1, NRT2 e NAR2, famílias de transportadores de nitrato; e NAR1, família de transportadores de nitrito [11]. Uma vez transportadas essas fontes são reduzidas a amônio e direcionadas ao metabolismo de N pela via GS-GOGAT. A redução de nitrato a nitrito é realizada pela enzima nitrato redutase (NR) no citoplasma. A redução de nitrito a amônio é catalisada pela enzima nitrito redutase (NiR) e acontece no estroma do cloroplasto [7,37]. Existem quatro formas da enzima glutamina sintetase (GS), duas citoplasmáticas e duas plastidiais; duas formas plastidiais da enzima glutamina:2-oxoglutarato aminotransferase (GOGAT), sendo uma NADH e outra Fdred.-dependente [7,37]. As fontes inorgânicas de N são preferenciais pelo fato de seu transporte ocorrer passivamente, após a assimilação dessas moléculas, as mesmas são assimiladas integralmente no metabolismo de compostos nitrogenados na célula [38,54,55].

O transporte de fontes orgânicas de N como ureia, por sua vez, ocorre através de mecanismos ativos. A ureia possui transportadores específicos, codificados a partir da família gênica DUR3, para os quais há três isoformas de transportadores específicos de ureia [52]. O transporte de ureia por essa via é sódio dependente e ativo. Este processo encontra-se ausente ou menos ativo quando outras fontes de N, em especial amônio, estão presentes [56–58]. O catabolismo de ureia em C. reinhardtii é realizado pela enzima ureia amidoliase (UAL-ase), que requer ATP, bicarbonato e íons de magnésio (Mg2+), biotina e cátions univalentes (K+, Na+, ou NH4+) para sua ativação [56]. A UAL-ase apresenta atividade de ureia carboxilase, catalisando a condensação de ureia e bicarbonato ATP-dependente, produzindo alofanato [56,59]. Em seguida, o alofanato é degradado pela enzima alofanato hidrolase, produzindo amônia e íon bicarbonato (HCO3-) [42,56,59,60]. Esta enzima é um complexo proteico, codificado pelos genes DUR1 e DUR2, que é ativado na ausência de amônio e presença de ureia ou acetamida [56]. Após a conversão

da ureia em amônio, o N proveniente da molécula pode ser assimilado via GS-GOGAT [56,58]. O CO2, resultante da hidrólise do alofanato, pode ser reduzido a HCO3 pela enzima anidrase carbônica [61,62]. Assim, o carbono pode ser usado tanto na via fotossintética quanto pode permanecer no citoplasma e contribuir para a manutenção do pH intracelular [59,60,63]. Dessa forma, ao se utilizar fontes orgânicas de N como a ureia além do metabolismo de N, o metabolismo de C também é suplementado através da assimilação dessas moléculas [52,64,65].

Diante de diversas fontes nitrogenadas assimiláveis e da importância do fornecimento de N para o crescimento e metabolismo em microalgas de uma forma geral, diversas variações dessas na formulação de meios de cultivo são estudadas [42,66,67]. As variações de fontes e concentrações de N tem sido testadas em cultivos de Chlamydomonas, e de outras clorófitas e diatomáceas, na tentativa de identificar as melhores combinações para produção de biomassa e compostos de interesse comercial [38,44,68–71]. A avaliação de nitrato e ureia como fontes alternativas de N para microalgas já foi realizada em clorófitas e diatomáceas, esses estudos avaliaram o crescimento e padrão de acúmulo de compostos e tinham como principais objetivos o incremento na produtividade de biomassa, aumento da produção de lipídeos e a redução de custos dos meios de cultivo [41–43,46,72,73]. Em Chlorella e Scenedesmus, experimentos com ureia foram realizados para avaliar a produção e acúmulo de triacilgliceróis (TAG) visando produção de biodiesel [42,44,51]. Para C. reinhardtii relatos da ureia como fonte de N são da década de 1980, compreendendo sua degradação e compartimentalização, mas sem discutir os impactos do seu uso no metabolismo [29,65]. Apesar dos diversos estudos já realizados em relação à utilização de fontes diversificadas de N, a compreensão detalhada do metabolismo e fisiologia em resposta a essas fontes ainda carece mais estudos [29,74,75].

Assim, o presente trabalho teve como foco compreender o metabolismo de ureia como fonte de N em C. reinhardtii. Sendo a ureia uma fonte de N e C, o suprimento fornecido pela molécula poderia ser utilizado nas vias de metabolismo de C e estimular o acúmulo de compostos de reserva. A partir disso, objetivamos entender de que forma o metabolismo de C e N e a fisiologia de C. reinhardtii seriam impactados pelo fornecimento de ureia ou amônio sob as condições de autotrofia e mixotrofia durante as fases logarítmica e estacionária. Adicionalmente, buscamos compreender de que forma o C proveniente da ureia poderia ser diferencialmente empregado nas condições de autotrofia e mixotrofia. Esta tese está organizada em três capítulos sendo os dois primeiros escritos em Inglês e na formatação de artigo científico, e o terceiro consiste em um apanhado das principais conclusões do trabalho bem como as perspectivas relacionadas ao uso de ureia no cultivo de C. reinhardtii sob diferentes condições nutricionais. Para um melhor entendimento do trabalho uma síntese dos capítulos 1 e 2 é apresentada a seguir.

Capítulo 1. Ureia pode ser utilizada como fonte de carbono e nitrogênio para Chlamydomonas reinhardtii? A chave pode ser o impacto da condição nutricional na razão C:N

A ureia é um composto nitrogenado orgânico bastante estudado por sua abundância no ambiente. Sua utilização como fonte de N em cultivos de microalgas já foi descrita em estudos anteriores, inclusive para Chlamydomonas reinhardtii. No entanto, a compreensão sobre os impactos metabólicos da ureia como fonte de N nas diferentes fases de crescimento sob os pontos de vista bioquímico e fisiológico ainda não foram realizados. Assim, este estudo teve como objetivo compreender como o metabolismo de C. reinhardtii CC503 é alterado ao se utilizar ureia como fonte de N nas condições de mixotrofia e autotrofia. Para isso C. reinhardtii foi cultivada em condições mixotrófica e autotrófica com o fornecimento de ureia ou amônio como fontes de N. Para avaliar os

impactos das condições nutricionais e das diferentes fontes de N foram avaliados parâmetros bioquímicos, fisiológicos e de crescimento em dois pontos da fase logarítmica e dois pontos da fase estacionária. A diferença entre as condições nutricionais de mixotrofia e autotrofia em C. reinhardtii já eram descritas anteriormente. Nossos experimentos confirmaram a diferença entre acúmulo de biomassa nas condições de autotrofia e mixotrofia, porém a distribuição temporal das fases logarítmica e estacionária não variaram em relação à condição nutricional. Em condições de mixotrofia, o suprimento orgânico de C proveniente do acetato tem impacto na aceleração do crescimento celular gerando incremento no número de células e biomassa, independente da fonte de N disponibilizada, quando comparado a autotrofia. A variação dos teores de pigmentos, proteínas, aminoácidos e carboidratos totais foram responsivas às condições nutricionais. Por outro lado, o acúmulo de amido e lipídeos respondeu diferencialmente em relação às fontes de N ao longo das fases para condições nutricionais distintas, assim como os parâmetros fisiológicos fotossíntese e respiração. Na condição de mixotrofia o total de clorofilas se manteve estável, as concentrações de compostos tenderam a decrescer temporalmente, e não foram observadas diferenças expressivas no acúmulo de amido e lipídeos de acordo com a fonte de N ofertada. Na condição de autotrofia, por sua vez, observou-se expressivo aumento no total de clorofilas, manutenção das concentrações de proteínas e aminoácidos e decréscimo no total de carboidratos. Adicionalmente, as diferentes fontes de N em autotrofia impactaram o acúmulo de compostos, sendo o acúmulo de amido e lipídeos maior na presença de amônio. Outra diferença relevante foi o aumento da taxa de fotossíntese em condições de autotrofia e de respiração especificamente em condição autotrófica e fornecimento de ureia como fonte nitrogenada. Os resultados demonstraram que o C proveniente da ureia é utilizado no metabolismo da microalga e que a condição nutricional em que a mesma é cultivada afeta as formas de armazenamento de compostos, o crescimento e fisiologia. O suprimento

orgânico de C via acetato na condição de mixotrofia atenua as respostas à diferenciação de fornecimento de N via amônio ou ureia. Essa resposta pode ser relacionada à maior razão C:N provocada pela assimilação do acetato. Em condição autotrófica é possível analisar melhor os impactos da fonte nitrogenada e, no caso da ureia, de que forma o C proveniente da mesma impacta o metabolismo e fisiologia. Assim, confirmou-se o emprego de ureia como fonte de N não promove estresse por privação deste nutriente e que o C proveniente da molécula pode estar envolvido como fonte adicional de C e estar relacionada com os impactos da condição nutricional na razão C:N, alterando a proporção de acúmulo de amido e lipídeos e as respostas fisiológicas de taxa fotossintética e respiratória.

Capítulo 2. Condição nutricional afeta o uso de ureia como fonte de C e N em Chlamydomonas reinhardtii.

O potencial uso de ureia como fonte de N e C, além de sua abundante disponibilidade no ambiente e comercialmente, tornam os impactos metabólicos dessa fonte nos organismos amplamente estudados. A microalga modelo Chlamydomonas reinhardtii é utilizada em diversos estudos em função de sua fácil manipulação, farta bibliografia referencial e por possuir seu genoma bem anotado. Essa microalga é capaz de transportar e assimilar ureia como fonte de N e C. O emprego de ureia no metabolismo de C. reinhardtii pode apresentar respostas diferenciais em relação às condições nutricionais de autotrofia e mixotrofia com impactos no padrão de armazenamento e crescimento, conforme demonstrado anteriormente. Assim, este estudo objetivou inferir sobre os impactos metabólicos em condições mixotrófica e autotrófica ao cultivar C. reinhardtii CC503 com ureia e amônio como fontes de N, buscando entender de que forma essa microalga utiliza ureia como fonte de C e N e quais as alterações metabólicas fazem com que as respostas ao uso de ureia em diferentes condições nutricionais sejam diferentes. Para isto foram analisados dados bioquímicos, metabólicos e incorporação de

isótopos de C (13C - Ureia, 13C Acetato), em três tratamentos (TAP + Amônio, TAP + Ureia, TAP, sem acetato + Ureia), na fase logarítmica dos cultivos. Nossos resultados confirmam que o uso de ureia não representa privação de N para C. reinhardtii e que o C proveniente da molécula é usado neste metabolismo. O crescimento em condição de mixotrofia é bastante acelerado quando comparado à autotrofia, o que já era descrito anteriormente. Os resultados demonstraram que na fase logarítmica o cultivo com ureia representa acúmulo de lipídeos em mixotrofia e de carboidratos em autotrofia. A presença de C orgânico na forma de acetato estimularia atividade fotorrespiratória e este processo seria relacionado ao menor acúmulo de carboidratos e amido, em detrimento ao de lipídios em mixotrofia. Sugere-se que o carbonato resultante do catabolismo da ureia é direcionado aos mecanismos de concentração de carbono (CCM) e atua no suprimento de C para fixação pelo ciclo de Calvin-Benson e não diretamente no ciclo TCA, como ocorre com o acetato. Assim, estimulação do processo de fixação do carbono via CCM seria responsável por diminuir a velocidade do ciclo celular e reduzir o processo fotorrespiratório em condições mixotróficas, atenuando os efeitos oxidativos e seus impactos no metabolismo das microalgas.

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CAPÍTULO 1

TITLE: Can urea be a source of carbon and nitrogen for *Chlamydomonas reinhardtii*? The key may be the impact of the C: N ratio

Rinamara Martins Rosa [1], Marcelo Gomes Marçal Vieira Vaz [1], Regina Lopes dos Santos[1], Wagner L. Araújo[1], Antônio Galvão do Nascimento[2], Adriano Nunes Nesi [1*]

[1] Departamento de Biologia Vegetal, Universidade Federal de Viçosa, 36570-900 Viçosa, Minas Gerais, Brazil.

[2] Departamento de Microbiologia, Universidade Federal de Viçosa, 36570-900 Viçosa, Minas Gerais, Brazil.

e-mail adresses: rinamara.rosa@gmail.com (R.M. Rosa), marcelogmvvaz@gmail.com (M.G. Marçal Vieira Vaz), re.lp.st@gmail.com (R.L.dos Santos), wlaraujo@ufv.br (W.L. Araújo), agn08julho70@gmail.com (A.G. do Nascimento), nunesnesi@ufv.br (A. Nunes-Nesi).

Key words: Autotrophy, Carbon, Mixotrophy, Urea

ABSTRACT

The temporal metabolic impacts of urea supply as a nitrogen (N) source based on biochemical and physiological parameters in *Chlamydomonas reinhardtii* was not investigated up to this moment. This work aimed to answer how the metabolism of *C. reinhardtii* CC503 responds using ammonium and urea as N sources under mixotrophic and autotrophic conditions. For this, growth, biochemical and physiological parameters were evaluated in logarithmic and stationary phases under mixotrophy the total chlorophyll was stable, the concentrations of compounds tended to decrease over time, and no significant differences were observed in the accumulation of starch and lipids according to the N source offered. In the autotrophy condition, there was a significant increase in total chlorophylls, maintenance of protein and amino acid concentrations and a decrease in total carbohydrates. In addition, the different sources of N in autotrophy affected the accumulation of compounds, with the accumulation of starch and lipids greater in the presence of ammonium. It was also observed an increase in the rate of photosynthesis under autotrophy and breathing conditions specifically in autotrophic condition and supply of urea as N source. Our results demonstrate that C supply is an impacting factor in the temporal variation of biochemical aspects and that C from urea is important under autotrophic conditions. We hypothesise that the high C:N ratio in mixotrophy masks the effects of the carbon supply by urea through the inhibition of carbon concentrating mechanism.

INTRODUCTION

The impact of carbon (C) and nitrogen (N) ratio on the metabolism of organisms is a widely studied theme [1,2]. The balance of these elements is essential because they are the principal components of live organisms' structure [3]. The way to obtain nutritional sources in the environment is related to evolutive aspects and promove impact on metabolic responses [4,5]. Inorganic C can be assimilated by chemotrophy or, most commonly, by photoautotrophy including a considerable number of species capable of making this fixation [6,7]. Nitrogen, on the other hand, is fixed only by bacteria making other organisms dependent of this process to get available N forms [8–10]. All these particularities make the relation between the C:N ratio and their available sources specific to each organism. This specificity of C and N metabolism and the responses to different sources and nutrition modes are essential to understand the primary metabolism modulation [3,11]. Organic and inorganic C sources can be used by *C. reinhardtii* and it determines the nutrition mode of this organism. *C. reinhardtii* and several other species of microalgae are photoautotrophs eukaryotes able to fix inorganic C as CO_2, HCO_3^-, and CO_3^{2-} [7,12,13]. In addition, *C. reinhardtii* can use acetate as organic C source adjusting its metabolism to heterotrophic mode (organic C supply and absence of light) [14,15], autotrophic condition (presence of light) or mixotrophic condition (organic C supply and presence of light) [16,17].

In microalgae, the C fixation occurs via photosynthetic metabolism, which is similar to higher plants. The pathway for C assimilation in *C. reinhardtii* is the same as C3 photosynthetic carbon reduction (PCR) cycle in which CO_2 enters directly in the Calvin-Benson cycle, like C_3-type higher plants. Studies also report a C concentrating mechanism (CCM) that accumulate different forms of soluble inorganic C, that are used in conditions of C deprivation [18,19]. In presence of low CO_2 concentration the photorespiration process can also be observed in microalgae, meantime CCM mechanism

inhibit the oxygenase activity of RuBisCO reducing energy losses [20,21]. All of these mechanisms in microalgae have great efficiency allowing these organisms to grow under low or high CO_2 conditions and have an effective C assimilation pathway [19,21,22].

Organic compounds can also be used as C sources to *C. reinhardtii*. acetate has specific influxes mechanisms in this microalgae [23,24] and can rapidly be incorporated into acetyl-coA by one of two pathways: being catalysed by acetyl-CoA synthetase or in two steps via Acetate-phosphate by acetate kinase and phosphate acetyltransferase [15,25]. In mixotrophic conditions, the use of organic C and CO_2 fixation by photosynthesis occur concomitantly [16,25]. Recent studies revealed that mixotrophic conditions allow culture growth, production and accumulation of storage compounds of C [26–29].

N metabolism is largely studied due to its importance to growth and accumulation of storage compounds. Just like C, N containing compounds can be assimilated as inorganic or organic forms [9,30]. In *C. reinhardtii* the preferential N sources are determined by the energy expenditure in the influx process, sources that can assimilate passively are preferential than active processes [21,31]. The mechanisms of N uptake involve membrane transporters specific for the N source and form to be transported [32,33]. Both organic and inorganic sources have specific systems that are responsive to endogenous concentrations of these substrates, according to the cell's metabolic capacity [33–35].

Recent metabolic studies have used the combination of C and N sources to study the compounds produced and their impact on storage [29,35,36]. These studies have already demonstrated that the growth of *C. reinhardtii* stands out when they are grown in mixotrophic conditions regardless of light availability regime [29]. Additionally, it is known that in mixotrophy the preferential N source is ammonium [37]. However, under the same conditions, the use of urea as a N source does not impact growth and stimulates lipid accumulation in chlorophytes [38,39]. Despite of the positive aspects of urea use,

the effects of combined ammonium and urea supply on different nutritional conditions have not been fully studied.

Variations in the N proportions and the N sources offered in microalgae cultivation have been studied in an attempt to increase productivity or accumulation of metabolites of interest[35,40,41]. In chlorophytes such as *Chlorella* and *Scenedesmus*, experiments with urea and ammonium were carried out to evaluate the production and accumulation of triacylglycerols (TAG) aiming at biodiesel production [36,42–44]. Another recent study with *C. reinhardtii* using urea as a N source demonstrated that the species has increased production of lipids and organic acids, suggesting that this source of N has an impact on the metabolism of C [39].

This study aimed to investigate the biochemical and physiological impacts on a *C. reinhardtii* cultivated under mixotrophic and autotrophic conditions using ammonium and urea as N sources. The results demonstrated how the microalgae metabolism is reprogramed along the growth phases by each treatment and how these changes generate the differences observed at the end of the cultivation in terms of storage compounds.

METHODS

Microorganism and culture media

Chlamydomonas reinhardtii CC503 is a registered model strain from Chlamydomonas Resource Center. The strain was cultivated in the mixotrophic medium Tris-Acetate-Phosphate (TAP) [45], this medium was adapted to be used in mixotrophic and autotrophic conditions by removing of acetate. The culture was cultivated under 24±2 ºC, photoperiod of 24:0 (light: dark) with a light intensity of 150 µmol photons $m^{-2}s^{-1}$, and constant shaking at 110 rpm. To produce inoculum for experiments the cells were cultivated for three days in the conditions described above. Afterwards, the cells were

115 centrifuged (13000 *g*, 5 min), and re-suspended in TAP medium without N (TAP-N) for
116 12 hours to deplete intracellular N[45].

117 In this study we test four different growth conditions: (i) complete TAP medium (TAP
118 Ac + Am); (ii) TAP medium with acetate and urea (TAP Ac+ Ur); (iii) TAP medium
119 without acetate and with ammonium (TAP Ac - Am); (iv) TAP medium without acetate
120 and with urea (TAP Ac- Ur). The media with acetate used pattern concentration of TAP
121 (1mL $L^{-1)}$. The concentration of N sources was 7mM of N, with proportional volumes to
122 ammonium and urea. The urea solution was sterilized by filtration and afterwards added
123 to sterile culture medium. All the solutions and medium were prepared using analytical
124 purity reagents.

125 **Culture conditions and growth parameters**

126 The experiments were performed in 125 mL Erlenmeyer flasks with 50 mL of useful
127 volume with initial cell density of $1x10^5$ cel·mL^{-1} and 5 repetitions per treatment. The
128 growth conditions were the same of inoculum production described above. For the
129 characterization of cell growth in different media and selection of sampling points we
130 performed growth curves for each treatment. The cellular growth was monitored daily by
131 absorbance on 750 nm in spectrophotometer (UVM 340, AsysHitech) for 6 days [46].
132 Based on the obtained growth curves we selected four points to collect samples as
133 indicated in the Fig. 1A, E: at logarithmic phase Log 1 (24h), Log 2 (48h); and stationary
134 phase Sta 1 (72h), Sta 2 (120h).

135 The morphological parameters shape, size and replication pattern were registered under
136 light microscopy. It was used the microscopy Olympus CX40, USA in 40x objective
137 (LCA chN) using SC30 capture system and Olympus Cell F software. The pictures were
138 processed with the free software ImageJ to calculate the cell area and volume [47,48]. To
139 determine the cell number the samples were fixed with Transeau solution [49] and the

cells counted with a haemocytometer (Neubauer chamber – New optics) under light microscopy (Olympus, CX40. USA).

The dry weight determination was performed by filtration of 10mL in nitrocellulose membranes 0,45 μm (Millipore®). These data were used to determine specific growth rate (μ), generation time (G2), mass per cell and specific growth rate at each growth phase as previously described [50].

Biochemical characterization

The biochemical analyses were carried out in samples collected in the selected time points defined above (Log 1, Log 2, Sta 1, Sta 2). To preserve the metabolites integrity, we collected 2mL of culture, centrifuged at 11200 g for 15min at 4ºC. The resulting supernatant was discarded and pellet immediately frozen in liquid nitrogen. The samples were stored at -80ºC until further analyses.

The sample was processed by a serial extraction protocol as previously described [51]. The levels of chlorophylls *a* and *b* [52] and total free amino acids were obtained using the methanol-soluble fraction obtained from hot methanol extraction [53]. The methanol-insoluble fraction was used for determination of starch [34] and total water-soluble proteins content [54]. Total carbohydrates was extracted according with described protocol [55], substituting HCl for H_2SO_4 and quantified as described by Masuko et al. [56]. The total lipid content was evaluated using a selective fluorescence method with Nile Red as described by Chen et al. [57]. The results for chlorophyll *a* and *b*, total chlorophyll, proteins, amino acids, total carbohydrates and lipids were expressed in μg per mg per dry weight of biomass. The starch content was expressed in □mol of glucose per mg per dry weight of biomass.

Physiological parameters

In order to evaluate the photosynthetic behaviour of the *C. reinhardtii* CC 503 grown in different conditions, we measured the evolution of oxygen on a Clark electrode (Oxy-Lab, Hansatech) in all four selected time points. For that, the flasks containing the cultures samples were acclimated in the dark for 20 minutes before being introduced into the electrode chamber [71]. In the measurements, we used 2 mL of culture, 150 RPM agitation, temperature of 25ºC and light intensity varying from 0 to 500 μmol·photons $m^{-2}s^{-1}$, with intervals of 50 μmol photons·$m^{-2}s^{-1}$, with intervals of 2 minutes. The obtained values were normalized by dry biomass. The light curves generated were adjusted according to the non-rectangular hyperbole model [59] and the following parameters were estimated: respiration, light compensation point, maximum assimilation rate and light saturation.

Statistical analyses

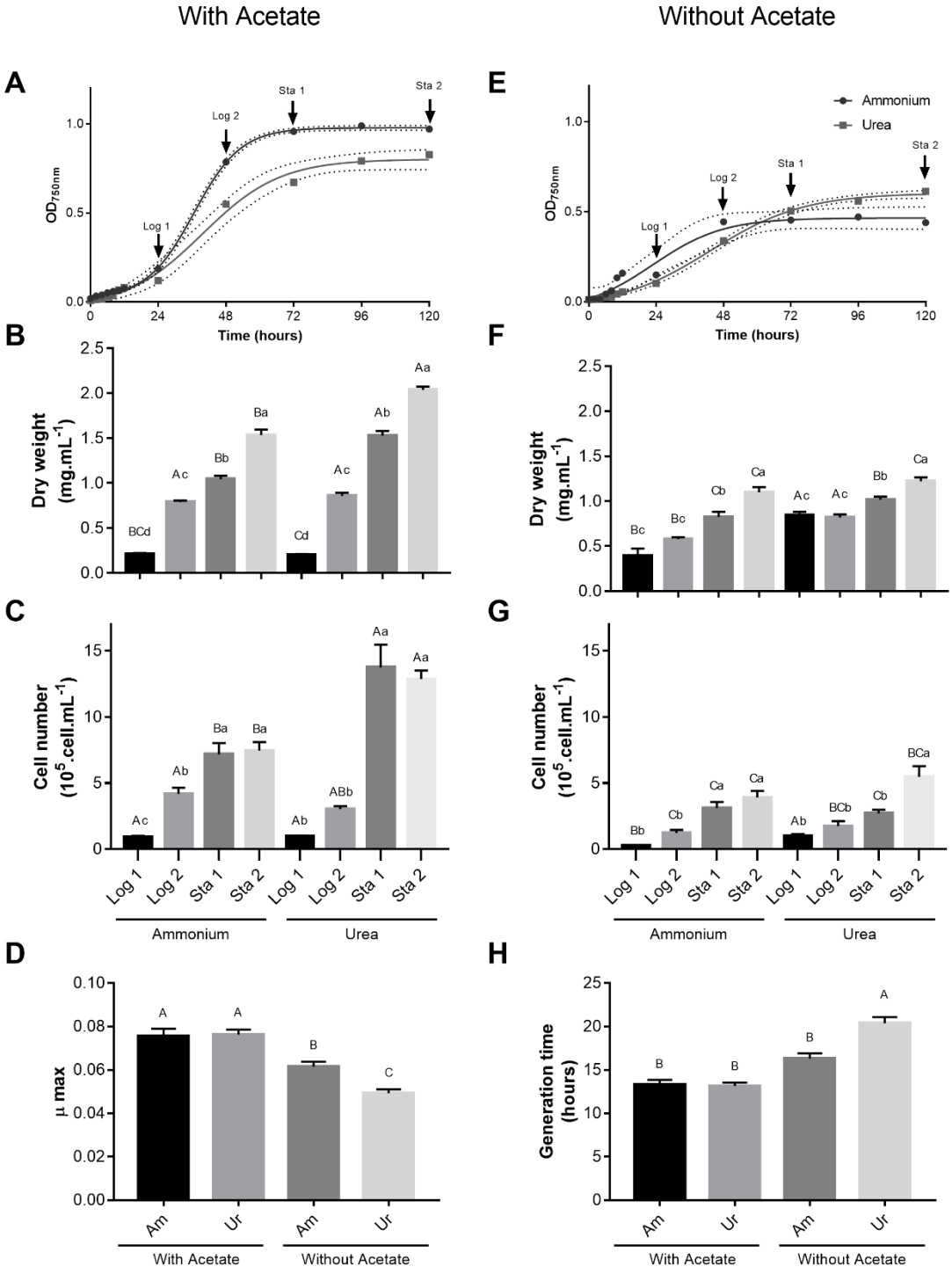
The experiments were performed and analysed following a completely randomized design with five replicates per treatment for each collection point. The values obtained for all variables were submitted to analysis of variance (ANOVA, $P<0.05$) followed by Tukey's test ($P\leq0.05$), using the SAS® software. The results from growth, biochemistry and physiological analyses were subjected to a principal component analysis (PCA) performed using the pcaMethods package in the software R [60].

RESULTS

Growth and biomass production

The analysis of growth parameters aimed to identify how cells grew and accumulated biomass over time. The cells were numerically quantified, as well as their morphological aspects (area and volume) and biomass. From these data, the growth parameters were calculated allowing other inferences about the behaviour of the culture over time. Growth curves based on optical density (OD) at 750nm indicated absence of differences during

the different growth phases. However, when comparing the growth in OD, it was demonstrated that there was greater growth in mixotrophy, compared to autotrophy. Considering the source of nitrogen in each nutritional condition, it is concluded that the medium containing ammonium in mixotrophy has greater growth compared to urea from Log 2 and that in autotrophy there are no growth differences between the sources of N. (Fig. 1A and 1E). The lower growth of the autotrophy media is confirmed by the dry weight and cell number data (Fig. 1F and 1G). Although the ammonium mixotrophic medium excelled in OD quantifications, the medium containing urea as N source led to a higher dry weight and number of cells in the Sta 1 and Sta 2 phases (Fig. 1B and 1C). Despite the indication of greater growth in the media under mixotrophic condition, the mass per cell was much higher in the Log 1 phase for media in autotrophy, mainly in absence of acetate and presence of ammonium treatment. These differences in cell mass between treatments were not observed from the Log 2 phase (Supplemental Fig. 1). Additionally, the impacts of growth, in different culture mediums, were showed in maximum growth rate (μmax, Fig 1D) and generation time (Fig. 1H), and demonstrated that there were no differences in growth rates between with ammonium and with urea treatments for mixotrophic condition. For the same parameters in the autotrophic condition, it was observed that with urea tends to grow more slowly, despite of that, these impacts were not noticed directly in dry mass and number of cells.



208 Figure 1 – Growth parameters of *C. reinhardtii* CC503 cultivated under different
209 conditions; TAP Ac+ and Ac- (mixotrophy and autotrophy) combined with different N
210 sources (ammonium and urea) with equimolar concentrations. Growth curves based on
211 Optical Density (OD) at 750 nm (A, E). Dry weight (B, F); cell number (C, G); growth
212 rate at Log phase (D); generation time (H). Values represent the average error of five

213 replications. Means followed by the same letters do not differ by the Tukey test ($P<0.05$),
214 uppercase letters compare conditions of mixotrophy and autotrophy at the same growth
215 phase between treatments and lowercase letters compare the growth phases isolated for
216 each treatment.

217 **Biochemical analyses**

218 The biochemical quantification of metabolites allows us to analyse how the organism is
219 responding to different treatments and how the cell composition is altered according to
220 the available C and N sources. Regarding total chlorophyll levels in mixotrophic
221 condition we observed lower values than those observed in autotrophic condition for both
222 sources of N from the Log 2 phase (Fig. 2A and 2E). Additionally, the chlorophyll
223 contents showed minor variation between the phases in the mixotrophic media and
224 tendency to increase in the Sta time points in autotrophy. This difference was observed
225 due to the reduction of Chl *a* and increase of Chl *b* along the phases in media with acetate,
226 while in the absence of acetate both Chl *a* and Chl *b* suffered an increase (Supplemental
227 Fig. 2). The impact observed in the pigment contents responds to the presence or absence
228 of acetate but did not differ significantly in relation to the supply of ammonium or urea.
229 The absolute values observed to total chlorophyll show that the contents were the biggest
230 ones in conditions without acetate, confirming the impacts of autotrophy. These
231 differences in chlorophyll contents suggest that under these conditions the microalgae
232 cells are not exhibiting any stress response because the chlorophyll *a*/*b* ratio do not alter
233 in media without acetate.

234 For the other N containing metabolites, the differences continued to be observed among
235 presence and absence of acetate. Protein content differ in Log 1 to other phases in media
236 with acetate and the greatest decrease is observed in the medium containing urea (Fig.
237 2B), media without acetate on the other hand kept the content stable during the growth

independent of the source of N (Fig. 2F). For the amino acids a similar response to proteins was observed, with variation between Log 1 phase and the others in media with acetate (Fig. 2C) and similar values in different growth phases for media without acetate not differing statistically between phases in this condition (Fig. 2G). However, the differences in protein and amino acids contents in media without acetate were clearly observed when protein/amino acids ratio was calculated, being the ratio similar and quite smaller for these treatments comparing to media with acetate (Fig. 2D and 2H).

Regarding the main C containing metabolites, we observed that starch contents were drastically different between phases for autotrophy and mixotrophy. The starch values increased over time in treatments without acetate from Sta 1 (Fig. 3A) despite the increases being observed for both N supplies, remarkable increase occurred for ammonium containing media. In contrast, from treatments without acetate the starch levels decreased on the phases Sta 1 and Sta 2 (Fig. 3D), with a clear reduction observed in the treatment with urea. Additionally, total carbohydrates were quantified and in this case treatments with and without acetate did not show differences in the accumulation pattern during the growth phases, but the storage of carbohydrates in treatments without acetate were about twofold larger than with acetate treatments (Fig. 3B and 3E), without differences in relation to N source. The content of lipids increased on Sta 1 and Sta 2 for treatments with acetate, being higher for with ammonium (Fig. 3C). For treatments without acetate this increment in lipids was observed only in Sta 2 to the condition of ammonium presence (Fig. 3F). The growth condition impacted the C sources storage changing the preferential class of storage metabolites. In mixotrophy conditions, the lipid contents was higher, in contrast starch and carbohydrates levels were reduced along the growth phases and showed lower values compared to autotrophic conditions. Under autotrophic conditions, higher values for sugars were observed and there was a tendency to accumulate starch during the time. Interestingly, the preferential sources of storage was

the opposite among mixotrophy and autotrophy but did not show different patterns related to the N supply. This data suggests that the use of urea do not represent a stress condition for *C. reinhardtii* in both mixotrophy and autotrophy growth conditions.

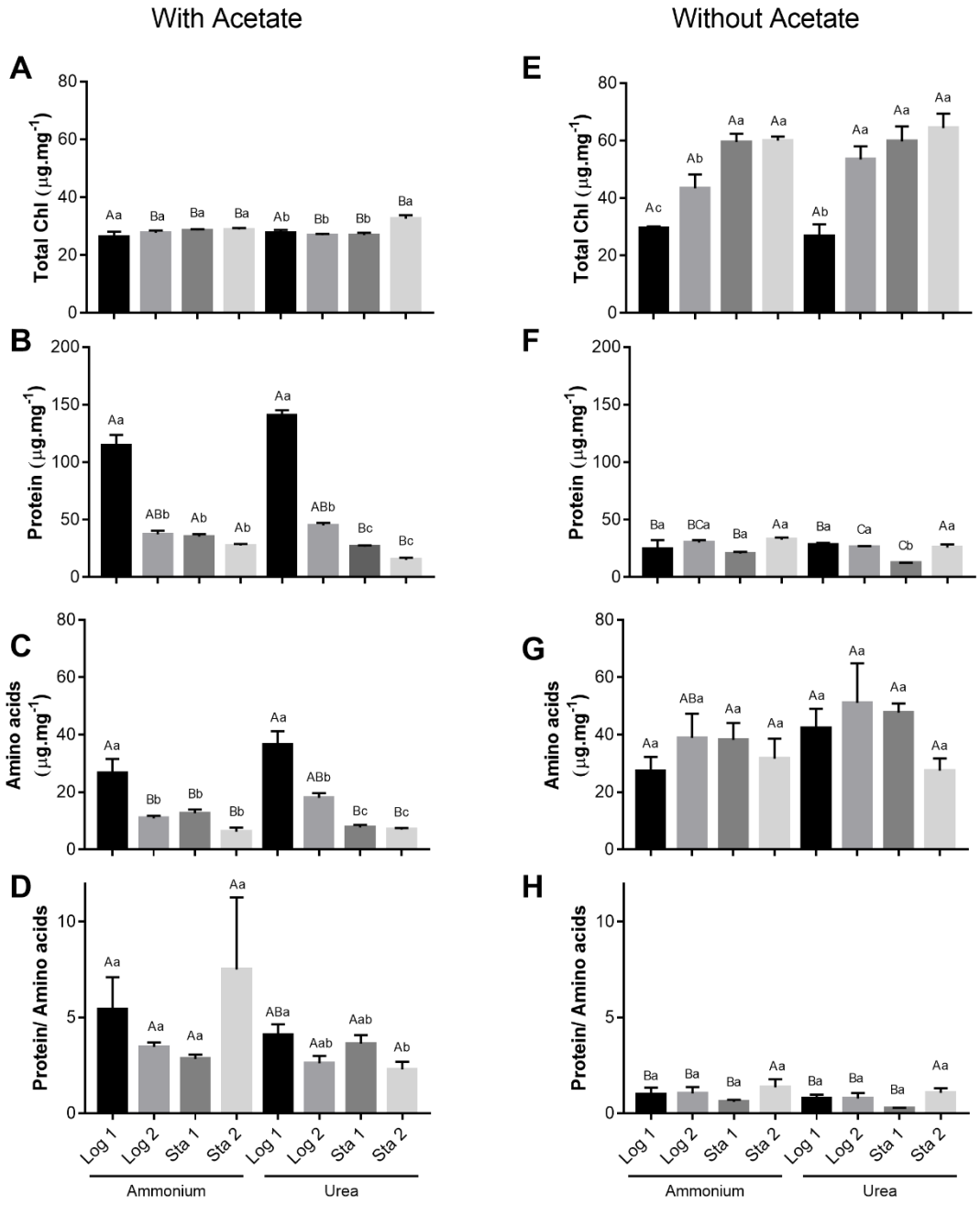


Figure 2 – N containing metabolites of *C. reinhardtii* cultivated under different conditions; TAP with and without acetate (mixotrophy and autotrophy) combined with different N sources ammonium and urea with equimolar concentrations. A, E total chlorophyll; B, F protein; C, G amino acids; D, H protein and amino acids ratio. Values

represent the average error of five replications. Means followed by the same letters do not differ by the Tukey test (P<0.05), uppercase letters compare conditions of mixotrophy and autotrophy at the same growth phase between treatments and lowercase letters compare the growth phases isolated for each treatment.

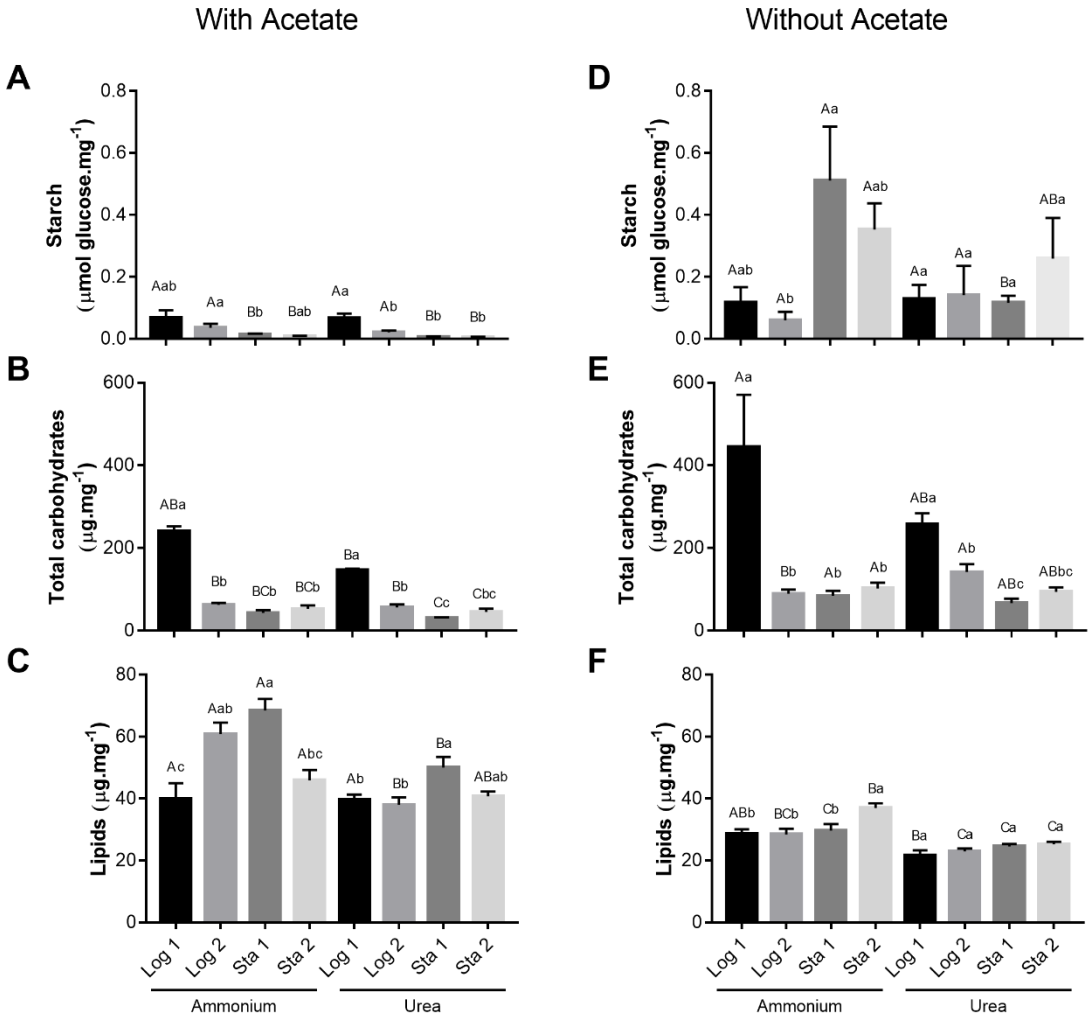
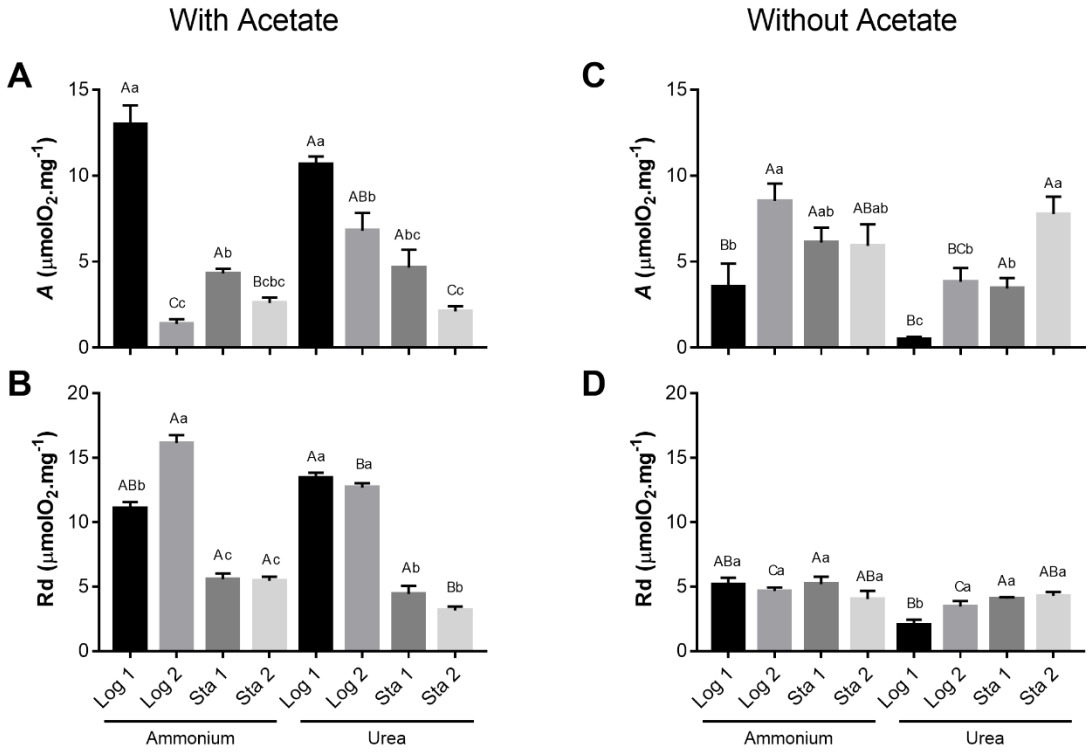


Figure 3 – Carbon containing metabolites of *C. reinhardtii* cultivated under different conditions; TAP with and without acetate (mixotrophy and autotrophy) combined with different N sources ammonium and urea with equimolar concentrations. A, D starch; B, E total carbohydrates; C, F lipids. Values represent the average error of five replications. Means followed by the same letters do not differ by the Tukey test (P<0.05), uppercase letters compare the same growth phase between treatments and lowercase letters compare the growth phases for each treatment

Physiological analyses

To understand the effect of the variations in the growth pattern and metabolites accumulation following urea supply under mixotrophic and autotrophic conditions, we performed physiological analyses. Despite not being observed contrasting differences in photosynthetic light compensation point and saturating light intensity between treatments, we observed differences between treatments and growth phases for O_2 evolution (Supplemental Fig. 4), that are responsible of impacts on photosynthetic and respiratory rates.

Under mixotrophic condition the photosynthetic rates decreased over the growth phases (Fig. 5A). Interestingly, this reduction was gradual on urea containing media and abrupt from Log 2 on ammonium containing media. The same behaviour was observed for respiration rates, though the rates did not change gradually between Log and Sta phases (Fig. 5B). On the other hand, in autotrophic condition, a trend of increase was observed for photosynthetic rates according to the growth phases, mainly in urea containing media (Fig. 5C). The respiration rates in these conditions was unaltered, but photosynthetic rates tend to increase on Sta phases in urea containing media (Fig. 5D). These results suggest that acetate supply impact on physiological responses in the same level as urea does.



299 Figure 4 – Physiological parameters of *C. reinhardtii* cultivated under different
300 conditions; TAP with and without acetate (mixotrophy and autotrophy) combined with
301 different N sources ammonium and urea with equimolar concentrations. A, C
302 photosynthesis; B, D respiration. Values represent the average error of five replications.
303 Means followed by the same letters do not differ by the Tukey test ($P<0.05$), uppercase
304 letters compare conditions of mixotrophy and autotrophy at the same growth phase
305 between treatments and lowercase letters compare the growth phases isolated for each
306 treatment.

307 **Multivariate analysis**

308 To understand the components that impact on responses observed in different growth
309 phases in each conditional nutrition on *C. reinhardtii* cultivation, the principal component
310 analysis (PCA) was performed (Fig.5). The main parameters related to group formations
311 are presented in loadings graphs on Supplemental Fig. 3. The score plots generated
312 indicated that the treatment grouping varies according to the growth phases. In the Log 1

and Log 2 phases, the acetate treatments are grouped according to biomass and protein and lipid accumulation. In contrast, the acetate treatments were separated influenced by physiological parameters, carbohydrate and starch levels. This clustering pattern is inverted in Sta 1 and Sta 2 where the separation of groups with acetate and the union of groups without acetate are observed. In these phases the separation of the groups is influenced by the accumulation of biomass in TAP with urea and proteins and lipids in TAP with ammonium. In contrast, the parameters that group the conditions without acetate are carbohydrates, lipids and chlorophyll. This analysis suggests that the source of N impacts treatments without acetate in the Log phase and treatments with acetate in the Sta phase. Additionally, we noticed that the impacts on main C containing compounds are mainly responsible for the change in clusters over time.

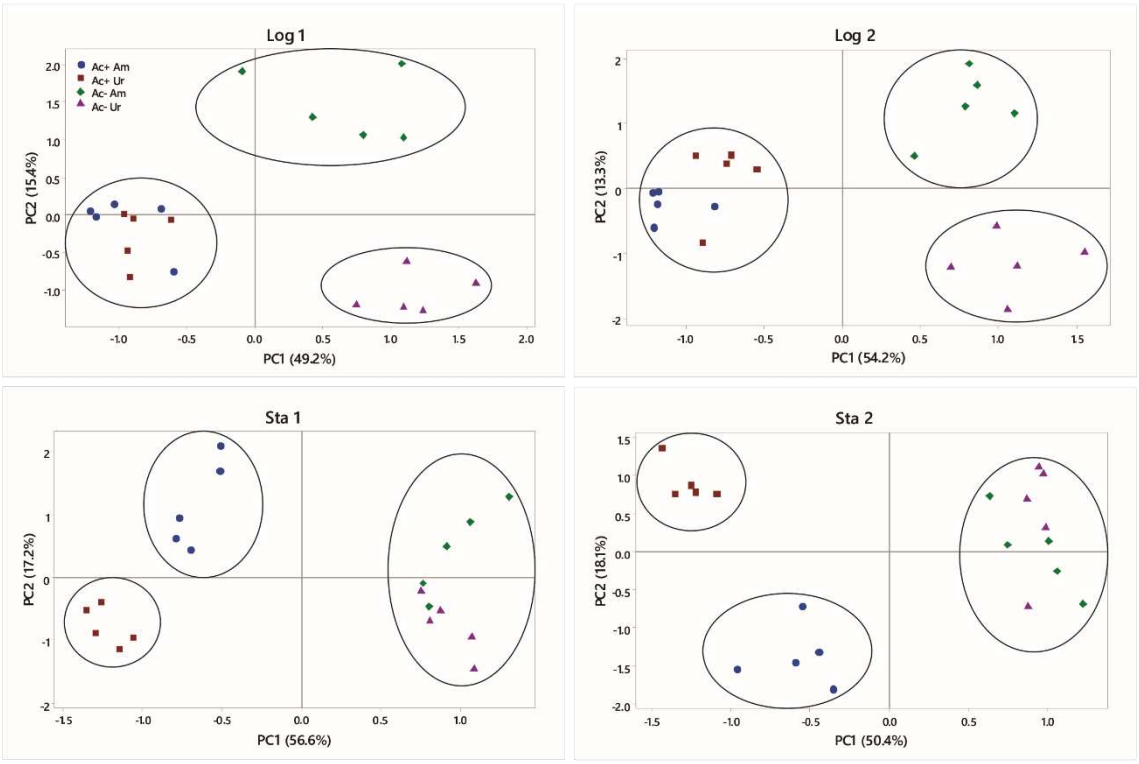


Figure 5 – Principal component analysis (PCA) score plot derived data of *C. reinhardtii* cultivated under different conditions; TAP with and without acetate (mixotrophy and autotrophy) combined with different N sources ammonium and urea with equimolar concentrations. Biochemical, physiological and growth parameters displayed in four

different growth phases; Log 1, Log 2, Sta 1, Sta 2. Explained percentage variance of principal components is shown individually in the graphs. Different templates observed for grouping of data indicate that metabolic components change with time impacting on responses to autotrophy and mixotrophy and the N sources. The graphs of PCA Loadings and principal component representations are shown in Supplemental Table 1.

DISCUSSION

Just as it happens with many organisms, microalgae cultures have a temporal variation in metabolic responses during their growth phases [61,62]. Variations can occur quickly within a single replication cycle (cell cycle) [36,63] or between phases when considering all the growth curve[64,65]. A study with the diatom *Skeletonema marinoi* monitored the metabolome during different growth phases and the results revealed clearly that the intracellular metabolites differ between exponential, stationary and declining phase[62]. It is known that the metabolites in different stages of growth are responsible to cultivation conditions and nutritional supply [11]. In this study we demonstrate how biochemical and physiological conditions alter according to the growth stages due to the autotrophic or mixotrophic metabolism with ammonium and urea (Fig. 6). Our results from PCA analysis indicate that variations in analysed parameters respond to growth phases and are mainly grouped according to conditions of autotrophy and mixotrophy (Fig.5). We observed that in Log 1 and 2 the mixotrophic medium are grouped mainly due to biomass, proteins and lipids. The same parameters are the main components that cause divergence between these medium in Sta 1 and 2. The opposite behaviour was observed for the autotrophy conditions, with carbohydrates and starch being the main components involved on groups separation at Log 1 and 2 and to the grouping at Sta 1 and 2 (Supplemental Fig. 3). These results reflect the impact of acetate metabolization in mixotrophic conditions at Log phases when the TCA cycle can be stimulated by acetate assimilation promoting intense growth and increase in storage compounds, this intense

354 metabolism will decrease over time by consumption of acetate. In the other hand,
355 autotrophic conditions have its storage of carbon compounds concentrated at Sta phases
356 reflecting the slower growth rate and the graduate assimilation of carbon using carbon
357 dioxide.

358 The accumulation of biomass and cell number in mixotrophic media is generally higher
359 when compared to the condition of autotrophy [27,29,64]. In studies with
360 *Chlamydomonas* this difference is attributed to the less complex metabolism of acetate
361 that stimulates the replication and growth [14,28]. These effects on growth were observed
362 when comparing medium without acetate (Fig. 1). The variation in N sources, however,
363 did not alter growth parameters in these cases. An important difference that was observed
364 was between mass per cell, which in absolute values was about 2 to 4 times greater in the
365 condition of autotrophy in phases Log 1 and 2 (Supplemental Fig.1C and 1G). This
366 difference is related to the impact of autotrophy in the cell cycle. In this condition the
367 replication time is higher and the increase in cell size during replication is also large [66].
368 Studies with *Scenedesmus*, *Chlorella* and *Chlamydomonas* reveal that in
369 photoautotrophy the cell-cycle progression needs to increase accumulation of compounds
370 in the early stages to support replication [67]. Despite the greater mass, the volume and
371 area of these cells did not change significantly either between phases or between
372 treatments (Supplemental Fig.1). In the mixotrophic condition three inputs of energy and
373 substrate occur concomitantly and the light, acetate and carbon dioxide are uptake
374 directing the flux of C assimilation and increasing rapidly de growth, biomass
375 accumulation and storage of C compounds. Similarly, to the C uptake by acetate, we
376 suggest that urea can be metabolized at the cell providing a small C supply that can impact
377 on C:N ratio and be a better N source than inorganic compounds.

378 Despite the similarities observed in the growth pattern between treatments, the
379 biochemical responses showed important variations. The total chlorophyll content

remained balanced between the times in the mixotrophic cultivation while gradually increasing in the autotrophic cultures (Fig 3A and 3E). However, the Chl *a/b* ratio decreased under mixotrophy and remained unchanged under autotrophy (Supplemental Fig. 2C and 2F). The Chl *a* decrease and Chl *b* increase temporally in mixotrophy impacting the Chl *a/b* ratio, is reported in the literature that this process can be occur in consequence of N starvation or shading condition [68–70], additionally when acetate is provided photosynthesis and Chl content can be impacted similarly to stress conditions despite of the cycle of electrons produced due to acetate metabolization [71,72]. For the other nitrogenous compounds, similar protein levels (Fig. 4B, F) were observed between the conditions and variation in the amino acid (Fig. 3C and 3G) content, which was 2 to 5 times higher in the Sta 1 and 2 phases in autotrophic condition. The amino acid content is related to accumulation of energy storage compounds such as starch, some amino acids are involved directly in energy storage, as enzyme for metabolism adjustment or precursor of compounds [73]. Additionally, the protein/amino acid ratio (Fig 3D and 3H) was lower in the autotrophy condition compared to the mixotrophy condition. These results must be associated with greater expression and activity of enzyme complexes in the autotrophy condition, both to allow the efficient metabolization of N compounds and to supply the production of apparatus for C fixation [74,75]. What draws attention to the results is the fact that the N source available did not have significant impacts on these N metabolites between nutritional conditions, which reinforces the hypothesis that N stress does not occur due to the supply of urea.

The C compounds showed relevant impacts both in time between treatments and comparatively between nutritional conditions. The reserve pattern in *C. reinhardtii* is associated with stress conditions due to the C source, both the excess and the deprivation of it stimulate the accumulation of starch and carbohydrates [76–78]. In our results, both starch and carbohydrates had a higher accumulation under conditions of acetate absence

406 (Fig. 4D and 4E) compared to acetate presence (Fig. 5A and 5B), and the starch still
407 showed an increase in the Sta 1 phases under autotrophy. Thus, the condition of
408 autotrophy may represent a deficiency in the C:N ratio for the strain causing the
409 accumulation of sugars[79,80]. Regarding this effect, in the condition of autotrophy with
410 urea as N source, the accumulation of starch and carbohydrates was lower in phases Sta
411 1 and 2. This data allows us to infer that the C from the urea molecule contributes to the
412 C: N balance mitigating the effects of C deprivation. On the other hand, the presence of
413 acetate stimulates the lipid synthesis in *C. reinhardtii* [25,81], which is associated with
414 the data obtained, once in a condition of mixotrophy the accumulation of lipids stands out
415 while the sugar content is impaired (Fig 4C and 4F).

416 The physiological impact of the treatments also highlighted the difference between
417 cultivation conditions. The decrease in photosynthetic rates under mixotrophy conditions
418 (Fig. 4A) goes against the data of the Chl *a/b* ratio, indicating that damage to the
419 photosynthetic apparatus occurs over time [21,82,83]. Despite this, respiratory rates (Fig.
420 4B) also decrease, suggesting that all metabolism is reduced in Sta 1 and 2. These results
421 may be related to the saturation of the growth conditions in the final stages in response to
422 the high growth rate observed in Log 1 and 2. The saturation of the growth causes the
423 reduction of the oxygen concentrations that impact on respiration rates[84,85]. In
424 contrast, the photosynthetic rates in autotrophy (Fig. 4C) showed an increase over the
425 phases, mainly in the presence of urea. The increase in Chl content contributes to this
426 greater photosynthetic efficiency. Regarding the performance of the treatment with urea,
427 it is possible to relate it to the presence of additional C from it. Urea, when assimilated,
428 is converted to ammonium and carbonate through the activity of the enzymes urea
429 carboxylase and allophanate hydrolase[86–88]. The microalgae *C. reinhardtii* has CCM
430 that can store the C from urea catabolism and make it available later[20,89]. Respiratory
431 rates in autotrophic conditions (Fig. 4D) remained lower compared to mixotrophy and

hardly differed between phases. The maintenance of stable respiratory rates is related to the maintenance of the metabolic conditions balance between the growth phases[90].

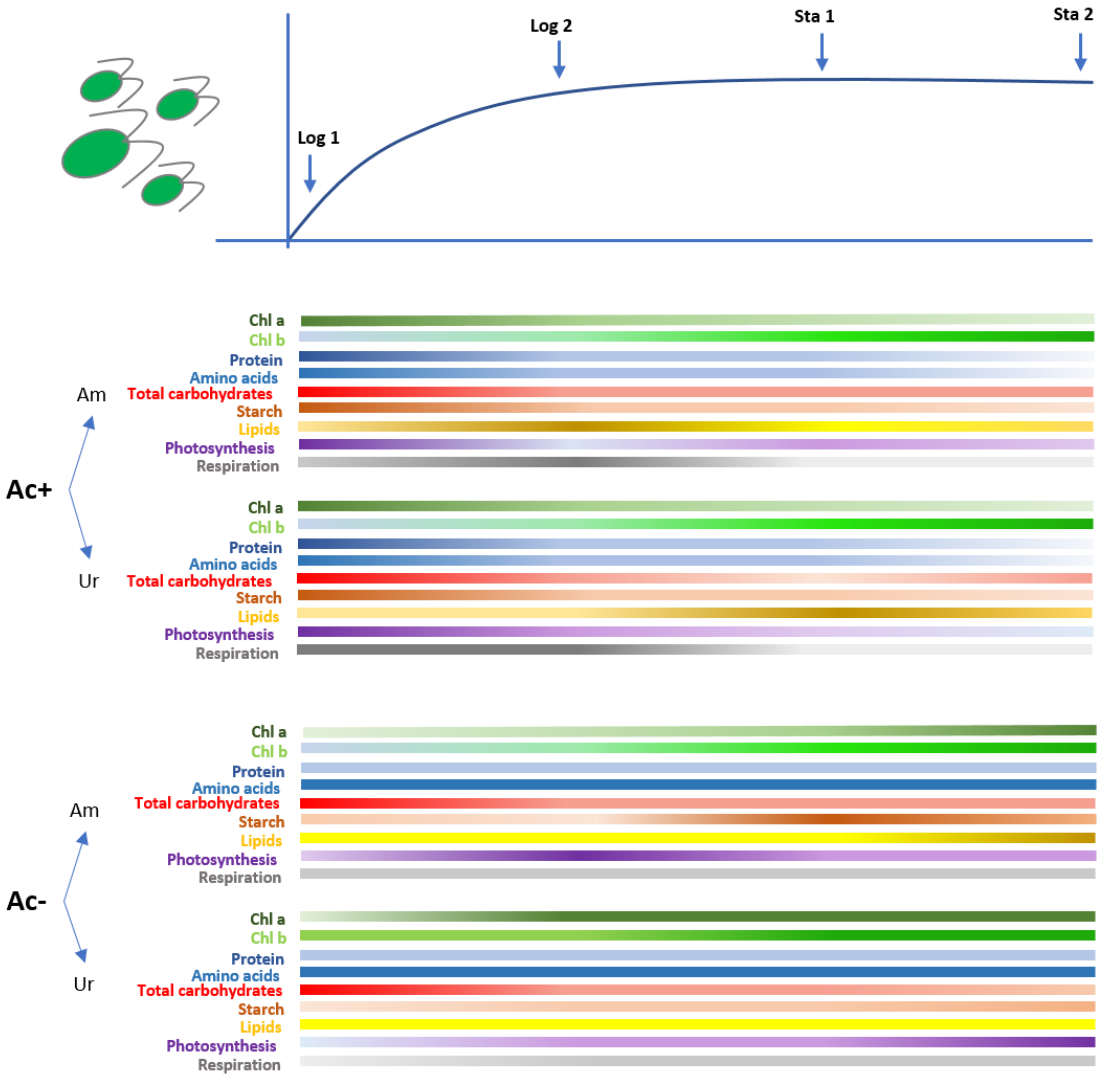
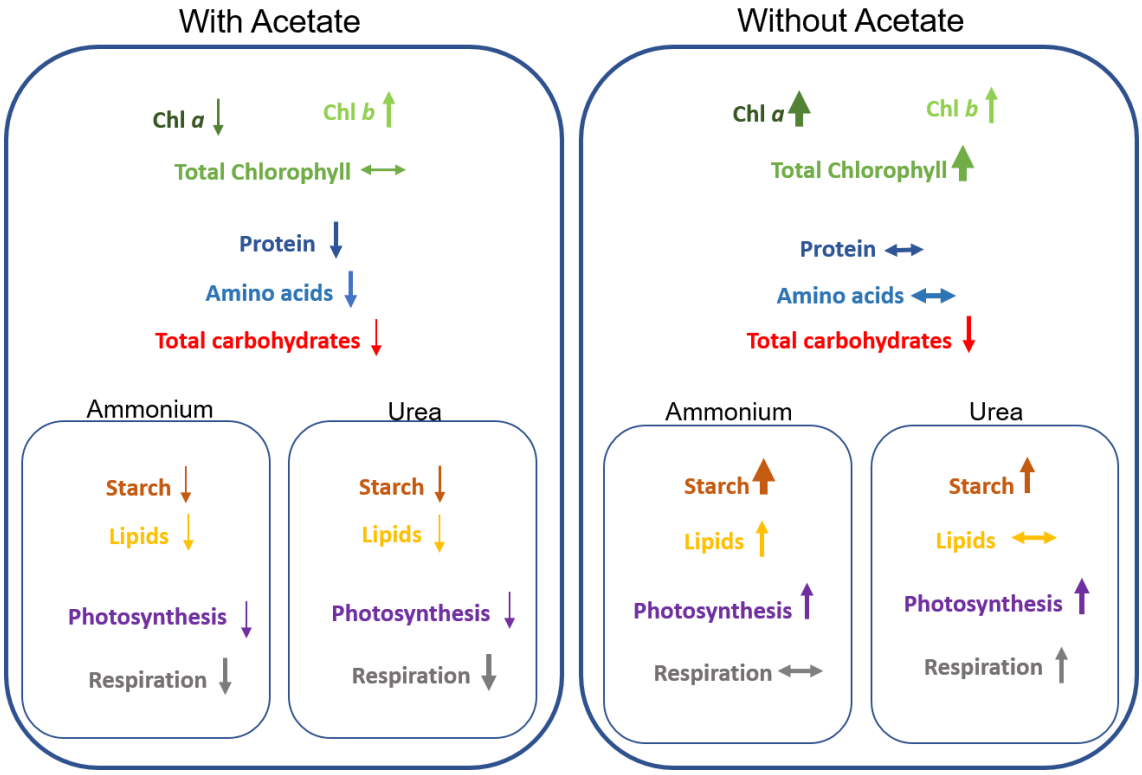


Figure 6 – Schematic representation of temporal changes in principal responsible parameters. The four culture conditions were separated in lines according to the caption. The parameters ware divided in colours and indicated on the figure. The intensity of colours represents the value of parameter, being the highest intensity the highest value and the same to the opposite. Colour scale variations ware determined according to the time analysed showed at the growth curve representation on the top.

CONCLUSIONS

Our data demonstrate that carbon supply is the most impacting factor in the temporal variation of biochemical aspects (Fig. 6). Under conditions of mixotrophy, the supply of organic carbon accelerates the growth rates of *C. reinhardtii* causing fast increase of cell number and biomass, saturating the culture medium with greater speed and generating impacts on metabolism. On the other hand, in autotrophy conditions, the culture remains without signs of stress for a longer time, continuing its photosynthetic activity and yielding the gradual accumulation of compounds as the cultivation stages progress. Additionally, we observed that responses to the supply of C from urea become clearer in the acetate absence condition, which makes us infer that the high C: N ratio in the acetate presence condition masks the effects of the supply of urea through the inhibition of CCM.

At the end of the cultivation it is possible to distinguish more clearly which aspects are impacted by the nutritional condition and the N source (Fig. 7). The N compounds vary according to the conditions with and without acetate, which reflects the absence of N deprivation in the treatments. The impacts related to the different N sources were observed in the physiological parameters and in the main reserve elements: starch and lipids. This difference highlights that the C from urea is being used in the metabolism of *C. reinhardtii*, causing significant impacts when in a condition with a lower C: N ratio in without acetate medium.



461 Figure 7 – Summary representation of principal responses according to the treatment. Up
462 arrows indicate increase, down arrows indicate decrease and vertical double arrows
463 indicate there were no difference. The thickness of the arrows was related to the values
464 of compounds or parameters evaluated.

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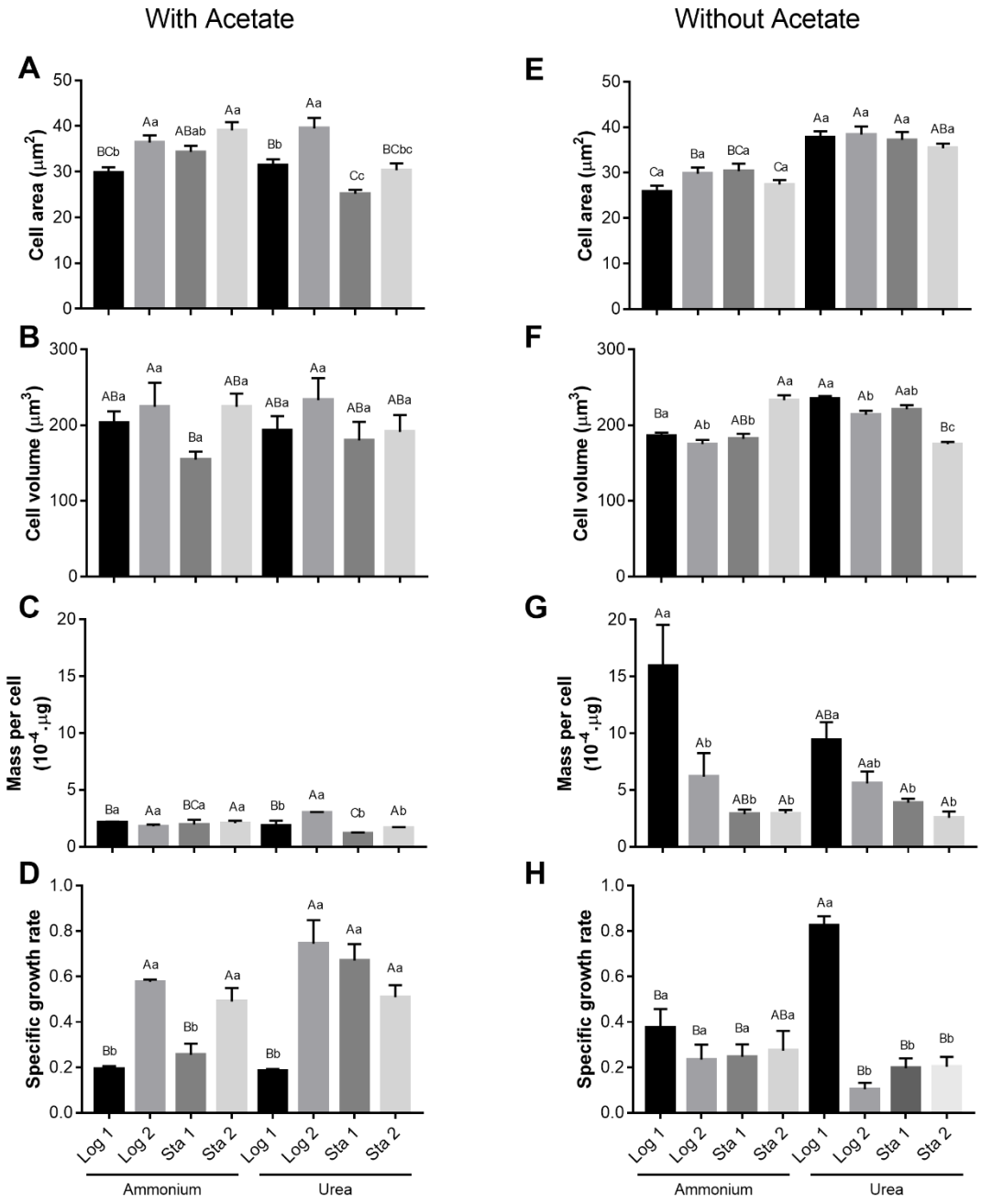
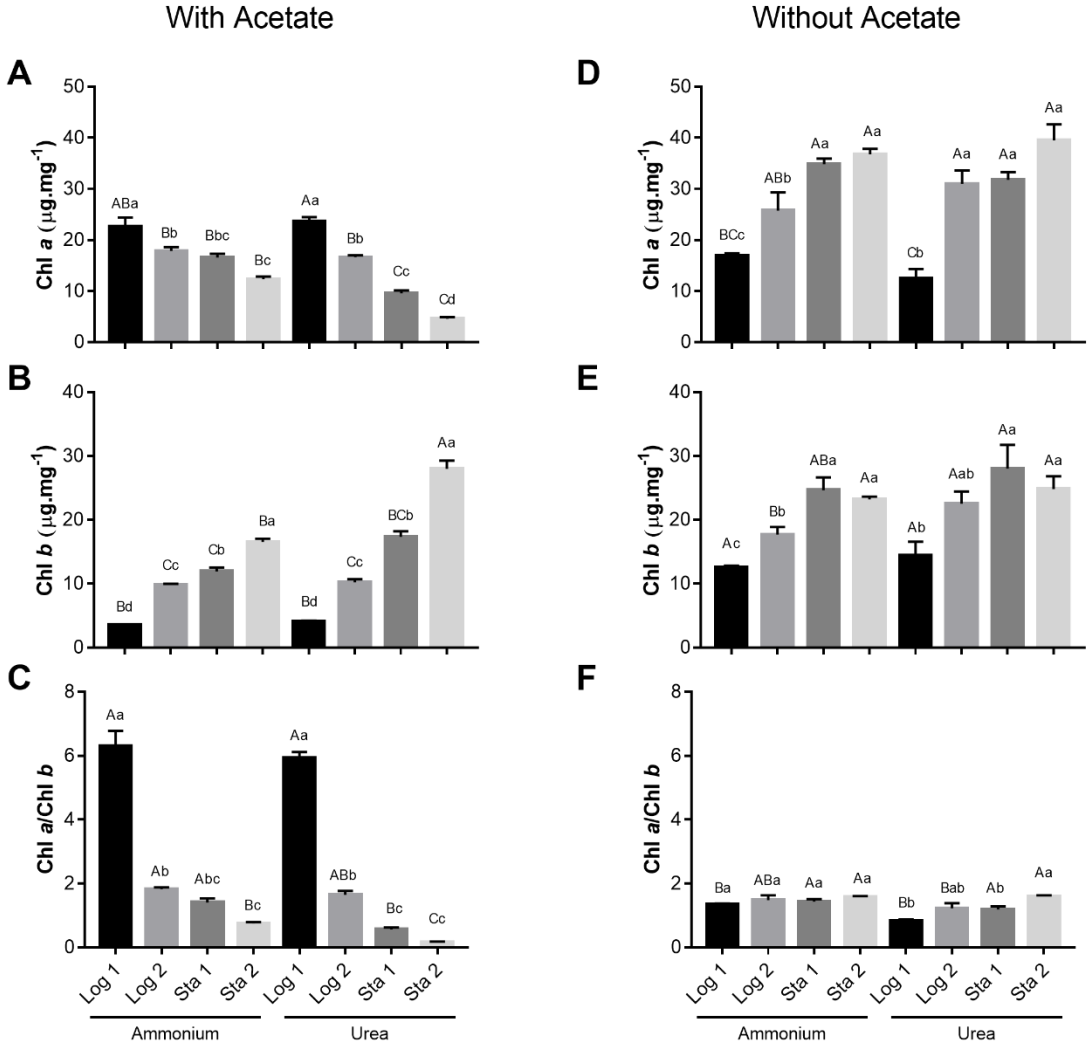
SUPPLEMENTAL FIGURES

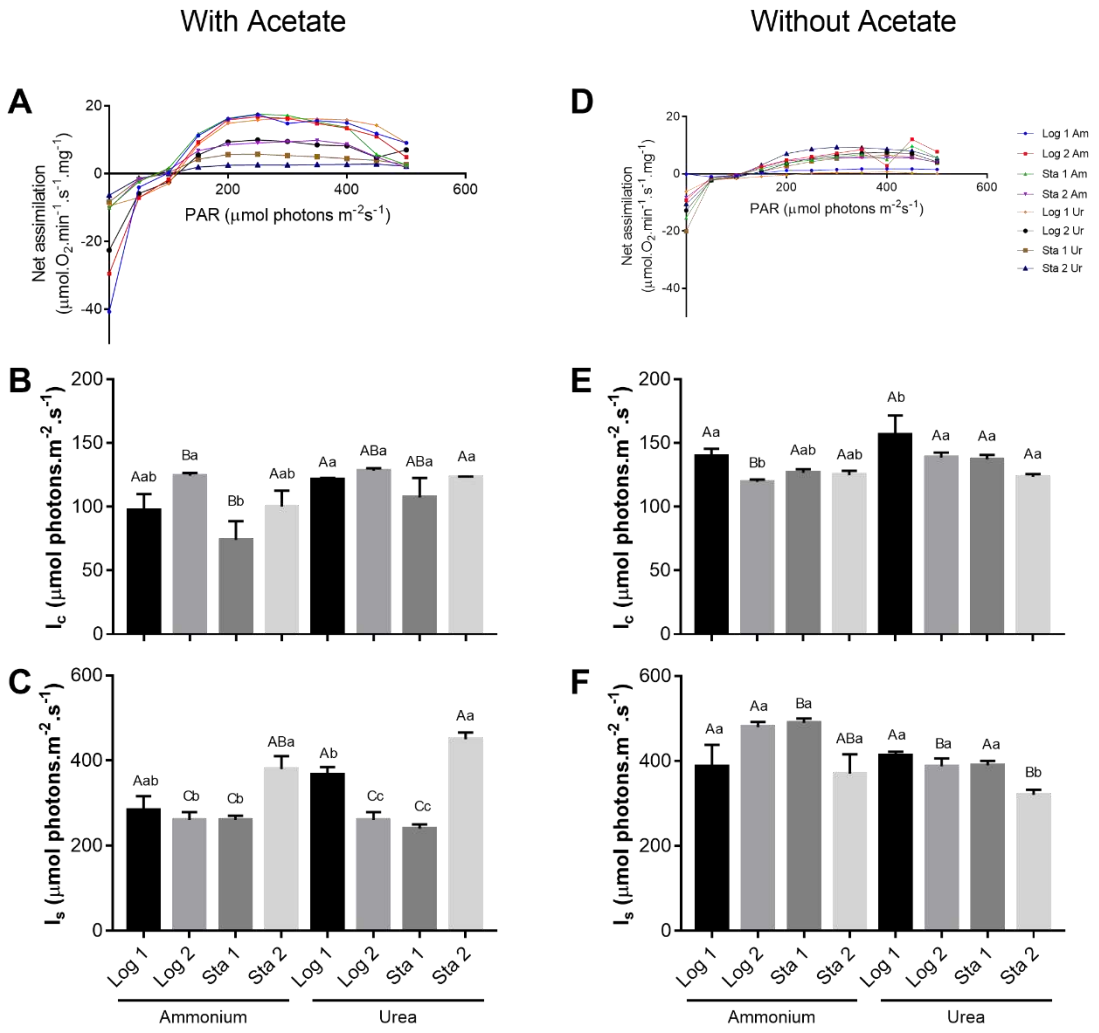


Supplemental Figure 1 – Growth parameters of *C. reinhardtii* cultivated under different conditions; TAP with and without acetate (mixotrophy and autotrophy) combined with different N sources ammonium and urea with equimolar concentrations. A, E cell area; B, F cell volume; C, G mass per cell; D, H specific growth rate. Values represent the average error of five replications. Means followed by the same letters do not differ by the Tukey test ($P<0.05$), uppercase letters compare conditions of mixotrophy and autotrophy

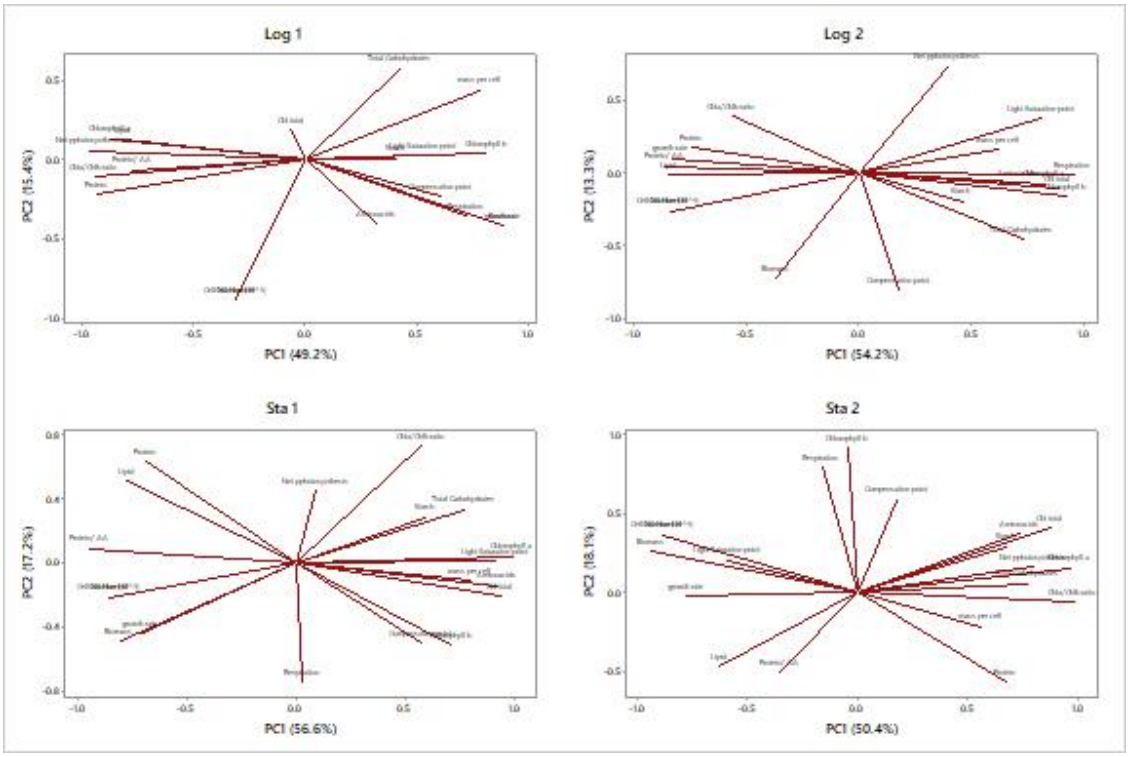
789 at the same growth phase between treatments and lowercase letters compare the growth
790 phases isolated for each treatment.



791 Supplemental Figure 2 – N containing metabolites of C. reinhardtii cultivated under
792 different conditions; TAP with and without acetate (mixotrophy and autotrophy)
793 combined with different N sources ammonium and urea with equimolar concentrations.
794 A, D chlorophyll a; B, E chlorophyll b; C, F chlorophyll a/chlorophyll b ratio. Values
795 represent the average error of five replications. Means followed by the same letters do
796 not differ by the Tukey test ($P<0.05$), uppercase letters compare conditions of mixotrophy
797 and autotrophy at the same growth phase between treatments and lowercase letters
798 compare the growth phases isolated for each treatment.



799 Supplemental Figure 3 – Physiological parameters of C. reinhardtii cultivated under
800 different conditions; TAP with and without acetate (mixotrophy and autotrophy)
801 combined with different N sources ammonium and urea with equimolar concentrations.
802 A,D light curve; B, E compensation intensity light; C, F saturation intensity light. Values
803 represent the average error of five replications. Means followed by the same letters do
804 not differ by the Tukey test ($P<0.05$), uppercase letters compare conditions of mixotrophy
805 and autotrophy at the same growth phase between treatments and lowercase letters
806 compare the growth phases isolated for each treatment.



Supplemental Figure 4 - Principal component analysis (PCA) score plot derived data of *C. reinhardtii* cultivated under different conditions; TAP with and without acetate (mixotrophy and autotrophy) combined with different N sources ammonium and urea with equimolar concentrations. Biochemical, physiological and growth parameters displayed in four different growth phases; Log1, Log2, Sta1, Sta2. Explained percentage variance of principal components is shown individually in the graphs. Parametrized data were Biochemical: Chlorophyll *a*, Chlorophyll *b*, Chl *a*/Chl *b* ratio, Total Chlorophyll, Total carbohydrates, Neutral lipids, Proteins, Amino acids, Protein, Protein/ Amino acids ratio and Starch; Physiological: Light compensation point, Light saturation point, Respiration and Net photosynthesis; Growth: Cell number, biomass, mass per cell and growth rate.

CAPÍTULO 2

TITLE: Nutritional condition affects the use of urea as C and N source in *Chlamydomonas reinhardtii*

Rinamara Martins Rosa [1], Regina Lopes dos Santos[1], Marcelo Gomes Marçal Vieira Vaz [1], Mariana Fonseca Machado[2], Wagner L. Araújo[1], Camila Caldana[3], Adriano Nunes-Nesi[1]

[1]Departamento de Biologia Vegetal, Universidade Federal de Viçosa, 36570-900 Viçosa, Minas Gerais, Brazil.

[2]Instituto de Biociências, Universidade Federal de Goiás – Regional Jataí, 75801-615 Jataí, Goiás, Brazil

[3]Max Plank Institute of Molecular Plant Physioplogy, 14476, Potsdam-Golm, Germany

e-mail adresses: rinamara.rosa@gmail.com (R.M. Rosa), re.lp.st@gmail.com (R.L.dos Santos) marcelogmvvaz@gmail.com (M.G. Marçal Vieira Vaz), mmachado21@outlook.com (M.F. Machado) caldana@mpimp-golm.mpg.de (C. Caldana); nunesnesi@ufv.br (A. Nunes-Nesi).

Key words: Carbon isotope, Carbon metabolism, Nitrogen metabolism, Urea

ABSTRACT

The abundant availability and application of urea, as well as its molecular structure capable of supplying carbon (C) and nitrogen (N), makes it widely studied in several organisms. The model microalgae *Chlamydomonas reinhardtii* is capable of assimilating urea as a source of C and N and it is known that the usage of this molecule as N source shows differential responses in autotrophy and mixotrophy conditions, with impact on storage and growth pattern. Our work aimed to investigate, through metabolite profiles and feeding experiments with ^{13}C, using ^{13}C-Urea and ^{13}C-Acetate, how the metabolism of *C. reinhardtii* is impacted under different nutritional conditions, with urea as a source of N. Our results shown that C from urea is metabolized by inorganic assimilation, differently of acetate but we can consider a mixotrophic condition for both C sources. When both organic sources are present in the crop, urea C may be responsible for attenuating changes in metabolism as well as stress caused by acetate metabolism, which is evidenced by the unchanged levels of chlorophyll, amino acids and the non-targeting metabolism for the production of lipids in the presence of urea. It is also concluded that, regardless of nutritional condition, C from urea catabolism is used in metabolism through the mechanisms of C fixation and both organic C supplies impact C:N ratio and can change or optimize storage compounds mechanisms.

INTRODUCTION

Among the various sources of nitrogen (N) used for microalgae cultivation, urea is one of the most common [1–3]. Recent studies addressed the impact of using this organic N source on the productivity and metabolism of microalgae [3,4]. Urea is naturally produced by bacteria, diatoms and animals through the ornithine-urea cycle [5,6]. In these organisms, it works as an alternative for maintaining homeostasis through the excretion of ammonium (NH_4^+) and carbonate (CO_3^{2-}), which together makes up the urea molecule (CH_4N_2O) [7–9]. Despite being an abundant source produced naturally by the excretion of animals, the synthesis of urea from industrial chemical processes is widely performed for the use of pure urea in areas such as pharmaceutical and agricultural [6].

In cyanobacteria several studies related to the role of urea in the eutrophication process and the stimulation of this molecule in the occurrence of blooms were performed [10,11]. Recently, it was demonstrated that under specific conditions urea can be used as a source of C and N and impact the metabolism of these organisms [12]. In plants, the mechanisms involved in the assimilation and use of urea have been intensively investigated because this source is the most used as fertilizer in agriculture, which makes the impacts of its effective use have direct responses on crop productivity[13]. These studies identified that, although there is a preference for the assimilation of N in the form of ammonium or nitrate after the decomposition of urea by soil microorganisms, despite urea transporters and enzymes capable of hydrolysing the molecule are present in plants [14,15]. In the case of microalgae, most studies on the use of urea involve modulation of starch and lipids production by supplying this source or using microalgae as a phytoremediation agent in eutrophic environments[1,16–18]. Although the absorption mechanisms of this molecule are well known and much has been studied about its impact on metabolism, the exact way in which this organic source of N is used by microalgae is not well described [4,19–21].

Due to the importance of C and N metabolism for microalgae productivity, the variation of sources and proportions of them is a frequently a topic of discussion [21,22]. Through them, it has already been demonstrated that under conditions of N deprivation, a metabolic reprogramming can promote the accumulation of lipids by directing C from photosynthesis to gluconeogenesis and glyoxylate pathways [23]. According to a study with *Chlorella zofingiensis*, N deprivation initially stimulates a massive production of starch and after a period of deficiency the accumulation of lipids[24]. In addition to the importance of available N concentration, the balance between the availability of C and N has a huge impact on metabolism. Similar to these stress results, the use of urea in the cultivation of microalgae also demonstrates the potential of the source for the accumulation of lipids, but without the inference that the microalgae is subjected to a situation of stress[21]. In mixotrophic conditions of growth using acetate as organic C supply, the accumulation of lipids is also stimulated by the uptake of C rapidly assimilated either the glyoxylate or tricarboxylic acid cycle [25,26].

In view of all these different results for the use and assimilation of N, the microalgae model *C. reinhardtii* has been an important tool to elucidate how the metabolic responses happen in each case [27]. Studies of the *C. reinhardtii* genome made it possible to list the transporters of N sources present in this alga. Ammonium (NH_4^+), nitrate (NO_3^-) and nitrite (NO_2^-) transporters are anchored in the plasma membrane and the chloroplast membrane [28]. Inside de *C. reinhardtii* cell the reduction of NO_3^- to NO_2^- is performed by the enzyme nitrate reductase (NR) in the cytoplasm. The reduction of NO_2^- to NH_4^+ is catalysed by nitrite reductase (NiR) and occurs in the chloroplast stroma [29]. Regarding N assimilation in *C. reinhardtii*, there are four isoforms of glutamine synthetase (GS), two cytoplasmic and two plastid; two plastid forms of glutamine: 2-oxoglutarate aminotransferase (GOGAT), one being NADH and the other $Fd_{red.}$-dependent [29,30].

The urea transport occurs through three isoforms of the specific transporter, encoded by the urea active transporter genes (DUR)[31], present in *C. reinhardtii*. The transport of urea through this route is sodium dependent and active. This process is absent or less active when other sources of N, especially ammonium, are present [4,32]. Urea catabolism in *C. reinhardtii* is carried out by urea amidoliase (UAL-ase), which requires ATP, bicarbonate and magnesium ions (Mg^{2+}), biotin and univalent cations (K^+, Na^+, or NH_4^+) for its activation. UAL-ase has urea carboxylase (UC) activity, catalyzing the condensation of urea and ATP-dependent bicarbonate, producing allophanate. Then, allophanate is degraded by allophanate hydrolase, producing ammonia and bicarbonate ion (HCO_3^-) [3,4,33,34]. This enzyme is a protein complex, encoded by the DUR1 and DUR2 genes, which is activated in the absence of ammonium and the presence of urea or acetamide. After the conversion of urea to ammoniac (NH_3), the N from the molecule can be assimilated via GS-GOGAT. CO_2, resulting from the hydrolysis of allophanate, can be reduced to HCO_3^- by the enzyme carbonic anhydrase. Thus, C can be used both in the photosynthetic pathway and can remain in the cytoplasm and contribute to the maintenance of intracellular pH [33,34]. In a complementary way a recent study has shown that the impact of using urea as a source of N varies according to the nutritional condition, being greater in conditions of autotrophy compared to mixotrophy[35].

Despite the various studies on the use of urea in *C. reinhardtii*, ranging from its assimilation, degradation and compartmentalization, to the impacts of its use on metabolism, the way in which urea C and N are incorporated into the metabolism and cause the results previously described is not yet well understood. This study aimed to understand the possible pathways by which urea is used as a C and N source in *C. reinhardtii* under mixotrophic and autotrophic conditions.

METHODS

Culture, strain and conditions

Chlamydomonas reinhardtii CC503 is a registered model strain from Chlamydomonas Resource Center. The strain was cultivated in the mixotrophic medium Tris-Acetate-Phosphate (TAP) [36], this medium was adapted to be used in mixotrophic and autotrophic conditions by removing of acetate. The culture was cultivated under 24±2 ºC, photoperiod of 24:0 (light: dark) with a light intensity of 150 µmol photons $m^{-2}s^{-1}$, and constant shaking at 110 rpm. To produce the inoculum for experiments the cells were cultivated for 3 days under the conditions described above. Then, the cells were centrifuged (5400 g, 15 min), and re-suspended in TAP without any N source (TAP-N) [36] and grown in this condition for 12 hours before applying treatments.

Experimental modelling

In order to identify how the C from urea would be assimilated under conditions of mixotrophy and autotrophy, experiments were planned with the supply of organic sources of C labelled with ^{13}C, a heavy isotope of C, acetate (Acetic acid, 2-13C, 99% - Cambridge Isotope Laboratories) and urea (Urea-13C, 99% - Sigma-Aldrich). In preliminary experiments three growth conditions were tested: (i) Standard TAP medium, with acetate and ammonium, (TAP Am+Ac); (ii) TAP medium with acetate and urea as N source (TAP Ur+Ac); (iii) TAP medium without acetate and with urea as N source (TAP Ur-Ac). In the media where acetate and ammonium were present, their concentration were the same as the standard TAP medium (17.48mM and 7mM, respectively). In the medium with urea as N source, the volume was calculated as equimolar of N related to ammonium. The urea solution was sterilized by filtration and afterwards added to the sterile culture medium. All the solutions and media were prepared using analytical purity reagents. Growth curves for each treatment were performed to

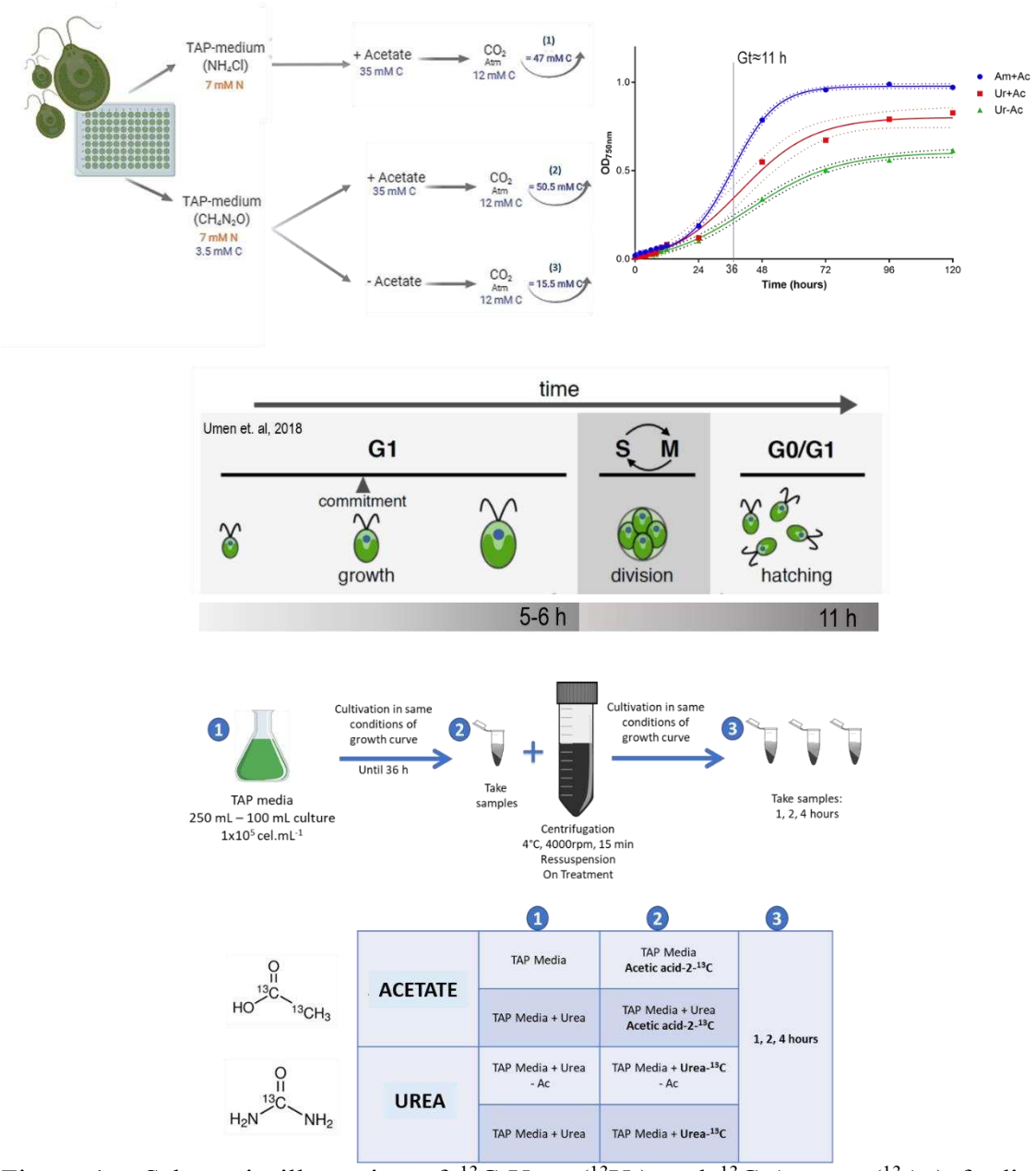
characterize the cell growth and select the time points along the growth phases that the treatment with C isotopes would be applied and the samples collected.

The cellular growth was monitored daily by absorbance on 750 nm (OD750nm) in spectrophotometer (UVM 340, AsysHitech) for 6 days [37]. From the generated growth curve, the sample collection point would be selected due to the shorter generation time. The selected time point was 36 h after the beginning of cultivation, where the generation time for all treatments was approximately 11 h (Fig. 1).

According to Umen et al. (2018) [38] during the replication process of *C. reinhardtii*, the assimilation of compounds and their metabolism before the division process occurs in about half the time destined for cell replication. Considering this information, the collections of the ^{13}C incorporation experiment were planned for a period prior to 5 h, which would be half the replication period under the culture conditions.

The experiments were performed in 250mL Erlenmeyer flasks with 100mL of useful volume with initial cell density of $1x10^5$ cel·mL^{-1} and three experimental units for each treatment. After 36 h of cultivation, the treatments were applied. The ^{13}C-Acetate or ^{13}C-Urea were applied in four different combinations: TAP with ammonium and ^{13}C-Acetate (Am+^{13}Ac); TAP with urea and ^{13}C-Acetate (Ur+^{13}Ac); TAP with ^{13}C-Urea and acetate (^{13}Ur+Ac); and TAP with ^{13}C-Urea without acetate (^{13}Ur-Ac). The experiment was carried out concurrently with the application of labelled substrates and unlabelled substrates, as controls. The cells were cultured up to 36 h, at each time point, 20 mL of culture were collected and the remaining volume was centrifuged at 4ºC, 5400 g, 15 min and resuspended in one of the treatment media described above. The flasks were resubmitted to the culture conditions and new sample collections proceeded after 1, 2 and 4 hours after the application of the treatments. The collected samples were used for

164 growth, biochemistry and metabolic analyses. The procedure and treatments applied are
165 described in detail in the diagram in Fig. 1.



166 Figure 1 - Schematic illustration of ^{13}C-Urea (^{13}Ur) and ^{13}C-Acetate (^{13}Ac) feeding
167 experiments in *C. reinhardtii*. The ^{13}C were applied in four different combinations: TAP
168 with ammonium and ^{13}C-Acetate (Am+^{13}Ac); TAP with urea and ^{13}C-Acetate (Ur+^{13}Ac);
169 TAP with ^{13}C-Urea and acetate (^{13}Ur+Ac); and TAP with ^{13}C-Urea without acetate (^{13}Ur-
170 Ac). A growth curve was performed to select the time of treatment application. The time
171 of 36 hours was selected according to the lower generation time to all treatments (≈11
172 hours). It was described by Umen et. al (2008) that incorporation of nutrients and its

metabolism occur in about half of generation time. Based on this data the intervals of samples collection were 1, 2 and 4 hours after applying the treatments. The figure details the experimental procedure for applying treatments and collecting samples. Culture conditions and the treatments were described in the table and details in Material and Methods.

Growth parameters

To determine the cell number the samples were fixed by Transeau solution [39] and the cells counted with a haemocytometer (Neubauer chamber – New optics) under light microscopy (Olympus, CX40. USA). The dry weight determination was performed by filtration in nitrocellulose membranes 0,45 μm (Millipore®). These data were used to determine mass per cell for each condition.

Biochemical analysis and metabolite profiling

For the biochemical analyses and metabolite profiling 20mL of cell culture was collected, submitted to metabolic quenching [40] and centrifuged at 11000 g for 15min at 4ºC, the supernatant was discarded and pellet immediately frozen in liquid N. The samples were stored at -80ºC until further analyses.

The sample processing used a serial extraction protocol as previously described [41]. The pigments chlorophylls *a* and *b* [42] and total soluble amino acids were conducted using the methanol-soluble fraction obtained from hot methanol extraction [43]. The methanol-insoluble fraction was used for determination of starch [44] and total water-soluble proteins content [45]. Total carbohydrates was extracted according with described protocol Teoh et al.[46], substituting HCl for H_2SO_4 and quantified as described by Masuko et al. [47]. The total lipid content was evaluated using Nile Red as described by Chen et al. [48]. The results for chlorophyll *a* and *b*, total chlorophyll, proteins, total free amino acids, total carbohydrates and lipids were expressed in μg per mg per dry weight

of biomass. The starch content was expressed as µmol of glucose per mg per dry weight of biomass.

For metabolite profiling an aliquot from the methanol-insoluble fraction was used. The samples were extracted as described above and analysed by gas chromatography-mass spectrometry (GC-MS) according to Machado et al (2016) [49] using an Agilent 6890 gas chromatograph-time-of-flight mass spectrometer (LECO Instruments, St. Joseph, USA). The chromatograms were exported from ChromaTof Software (version 3.25). Resulting chromatograms were processed in the program TargetSearch from the Bioconductor package in R software [50]. To normalize the results and obtain relative quantifications all the peak area was used internal standard (ribitol) and dry weight in sample.

Determination of isotope distributions

The samples treated with sources containing isotopes were collected and processed in the same way as the other samples described above. The resulting chromatograms were processed using TagFinder software. Analytes were selected manually using the TagFinder plug-in of the TagFinder software [51]and the reference library mass spectra and retention indices housed in the Golm Metabolome Database (http://gmd.mpimp-golm.mpg.de[52]. The GC-MS-TOF matrices were posteriorly processed using the Corrector software tool [53,54]. After correction, the ^{13}C incorporation percentages per metabolite obtained were used for the interpretation of the flow data.

Statistical analysis

The experiments were performed and analysed following a completely randomized design with five replicates for each treatment. The values obtained for all variables were submitted to analysis of variance (ANOVA, $P<0.05$) followed by Tukey's test ($P\leq0.05$), using the SAS® software.

RESULTS

Growth and biomass production

The evaluation of growth parameters showed that, despite having the same dry weight after 36 h of culture (Fig. 2C), the number of cells was significantly higher for media containing acetate (Fig. 2A). This variation in number of cells had an inverse impact on cell mass, which was significantly greater only for Ur-Ac (Fig. 2B). These data demonstrate that while the absence of acetate promotes a greater accumulation of biomass for replication, media containing acetate promote rapid replication in this phase.

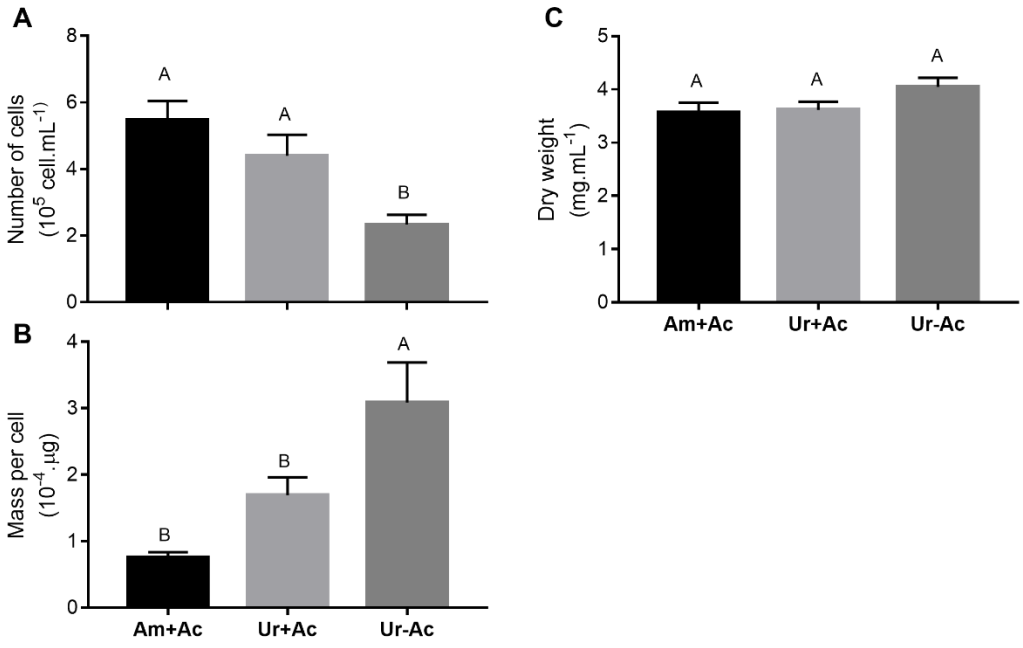


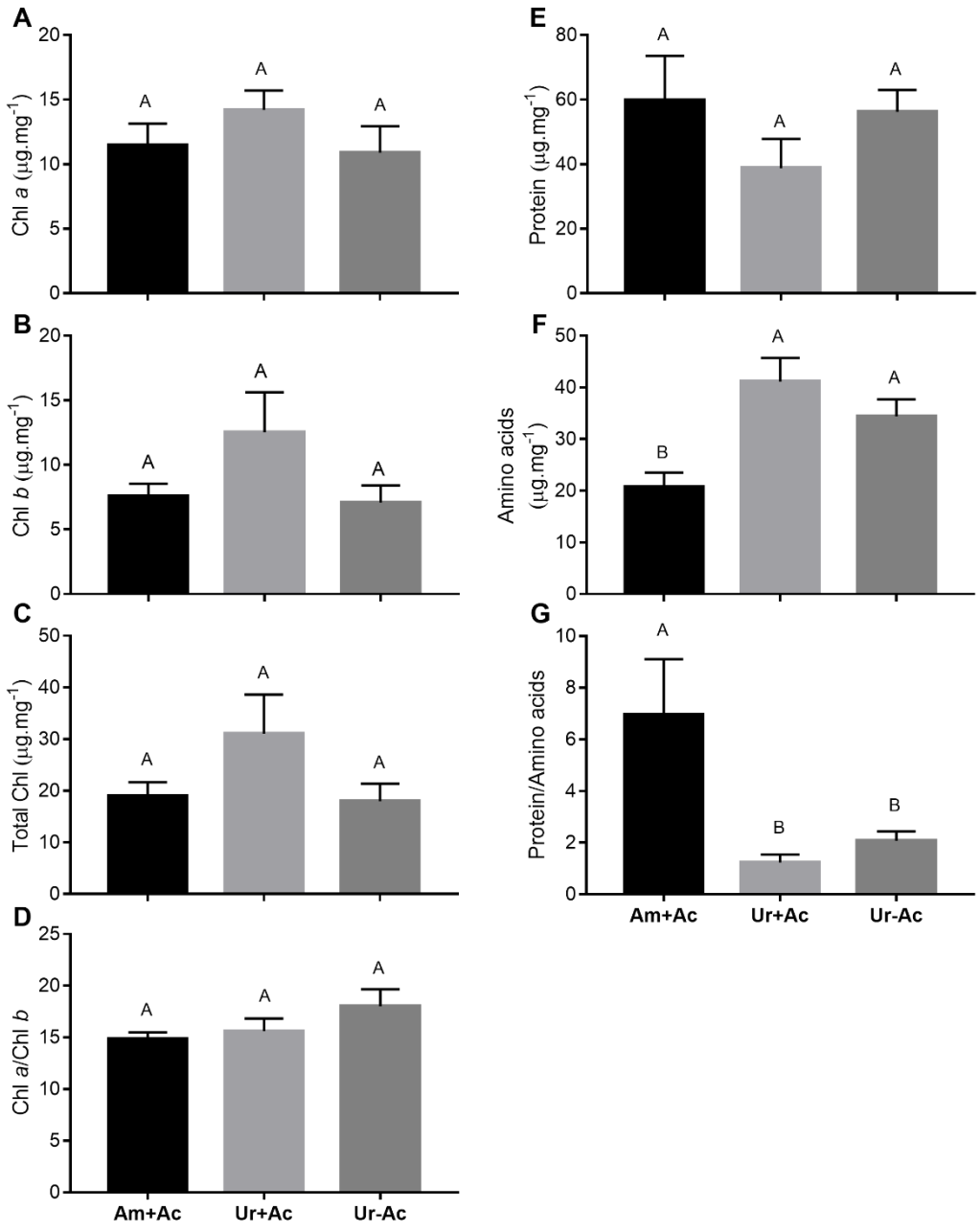
Figure 2 - Growth parameters of *C. reinhardtii* cultivated under different conditions. TAP medium supplemented with ammonium and acetate (Am+Ac); TAP medium supplemented with urea and acetate (Ur+Ac) and TAP with urea and without acetate (Ur-Ac). Dry weight (A); Mass per cell (B) and cell number (C). Samples were collected in the log phase in 36 h of culture. Values represent the average error of six replications. Means followed by the same letters do not differ by the Tukey test ($P<0.05$).

238 **Biochemical analyses**

239 The biochemical characterization of the treatments aimed to identify variations in the
240 main C and N compounds in the culture. The levels of chlorophyll *a* (Fig. 3A) and
241 chlorophyll *b* (Fig. 3B) did not show statistical differences between treatments, as well as
242 the values of total chlorophyll (Fig. 3C) and chlorophyll a*/b* ratio (Fig. 3D). These data
243 confirm that the culture conditions did not represent a stress situation for *C. reinhardtii*
244 since variations in the proportions of Chl are known to be associated with stress[55,56].

245 In a complementary way, the protein contents also did not present significant differences
246 between the treatments (Fig. 3E). The variation in N content was observed for amino
247 acids (Fig. 3F) where the media with urea as N source was higher than with ammonium.
248 This variation had an impact on the protein/amino acid ratio, making it much higher for
249 the treatment containing ammonium (Fig. 3G).

250 Additionally, the quantification of the main C containing compounds was performed. In
251 this case, the accumulation of starch (Fig. 4A) and total carbohydrates (Fig. 4C) did not
252 show differences between treatments. In contrast, the quantified lipid contents (Fig. 4B)
253 indicate that the accumulation of lipids was significantly lower in media containing urea.
254 These data indicate that the accumulation of lipids and amino acids in logarithmic phase
255 were more related to the N source offered than to the presence or absence of acetate.



256 Figure 3 – Nitrogen containing metabolites of *C. reinhardtii* cultivated under different
257 conditions. TAP medium supplemented with ammonium and acetate (Am+Ac); TAP
258 medium supplemented with urea and acetate (Ur+Ac) and TAP with urea and without
259 acetate (Ur-Ac). Chlorophyll *a* (A), chlorophyll *b* (B), total chlorophyll (C), chlorophyll
260 *a/b* ratio (D), protein (E), amino acids (F), and protein/amino acids ratio (G). Samples
261 were collected in the log phase in 36 h of culture. Values represent the average error of
262 six replications. Means followed by the same letters do not differ by the Tukey test
263 ($P<0.05$).

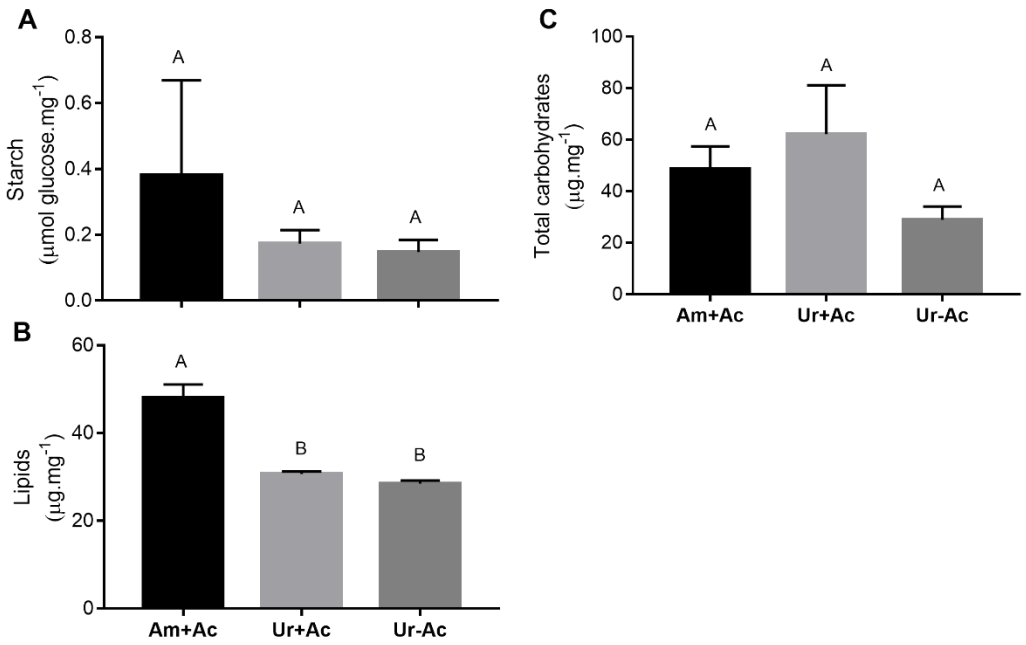


Figure 4 - C containing metabolites of *C. reinhardtii* cultivated under different conditions; TAP with ammonium and acetate (Am+Ac), TAP with urea and acetate (Ur+Ac) and TAP with urea and without acetate (Ur-Ac). Starch (A), lipids (B), and Total carbohydrates (C). Samples were collected in the log phase in 36 h of culture. Values represent the average error of six replications. Means followed by the same letters do not differ by the Tukey test ($P<0.05$).

Metabolite profiling

In order to describe the temporal metabolic alterations on treatments without addition of C isotopes we employed an established GC/MS based metabolite profiling platform. We considered the medium TAP Am+Ac to standard condition. This analysis revealed that at the stage when data were collected, the levels of sugars such as glucose, fructose and sucrose did not differ significantly between treatments. Differences in sugar content were observed for threonic acid and glycerol-3-phosphate, which decreased significantly in the treatments with urea, and opposite to 3,6-anhydro-L-galactose, where there was an increase significantly in urea treatments (Fig. 5).

Regarding the levels of organic acids, among the 16 metabolites identified, 12 showed a significant reduction in the Ur -Ac treatment. Malate, citrate and glycolate were reduced only in Ur -Ac, while α-ketoglutarate suffered an impact in both treatments with urea, decreasing significantly and gradually in Ur +Ac and Ur -Ac, compared to the standard condition. (Fig. 5). Despite the reduction observed in the total free amino acid levels for Am +Ac (Fig. 3F), most of the quantified amino acids were reduced in the treatments Ur +Ac and Ur -Ac. In contrast, beta-alanine, homoserine, serine and tryptophan were reduced in cells under Am +Ac condition (Fig. 5 and Supplemental Fig. 1). Interestingly, the levels of glutamine and glutamate, directly involved in the GS-GOGAT pathway, did not differ between treatments.

Isotope ^{13}C incorporation

The results of the ^{13}C isotope incorporation analysis indicated that C is incorporated from both sources, acetate and urea, was assimilated into different intermediates of the glycolysis, TCA cycle and amino acids metabolism (Fig. 6 and Fig. 7). Interestingly the percentage of label incorporation was clearly different for the two C sources. The C from these sources is used for both C and N metabolism, being identified in amino acids, their precursors and metabolites involved in central C metabolism amino acids (Fig. 6 and Fig. 7). We observed that the C incorporated from acetate had no impact due to the N source (ammonium or urea) for most of the identified molecules, with exception for valine and uracil. The incorporation of C from urea was altered by the absence or presence of acetate in the medium. Both C and N compounds were affected due to the cultivation conditions in these cases. The amino acids alanine, glutamic acid, valine and serine showed an increase in C from Ur -Ac. Similarly, organic acids such as citrate, glycerate, malate and succinate also exhibited incorporation of C from Ur -Ac over time. Together these results demonstrate that although the C from urea is assimilated in the same pathways as that

from acetate, nutritional conditions (autotrophy or mixotrophy) interfere with the way that C is used.

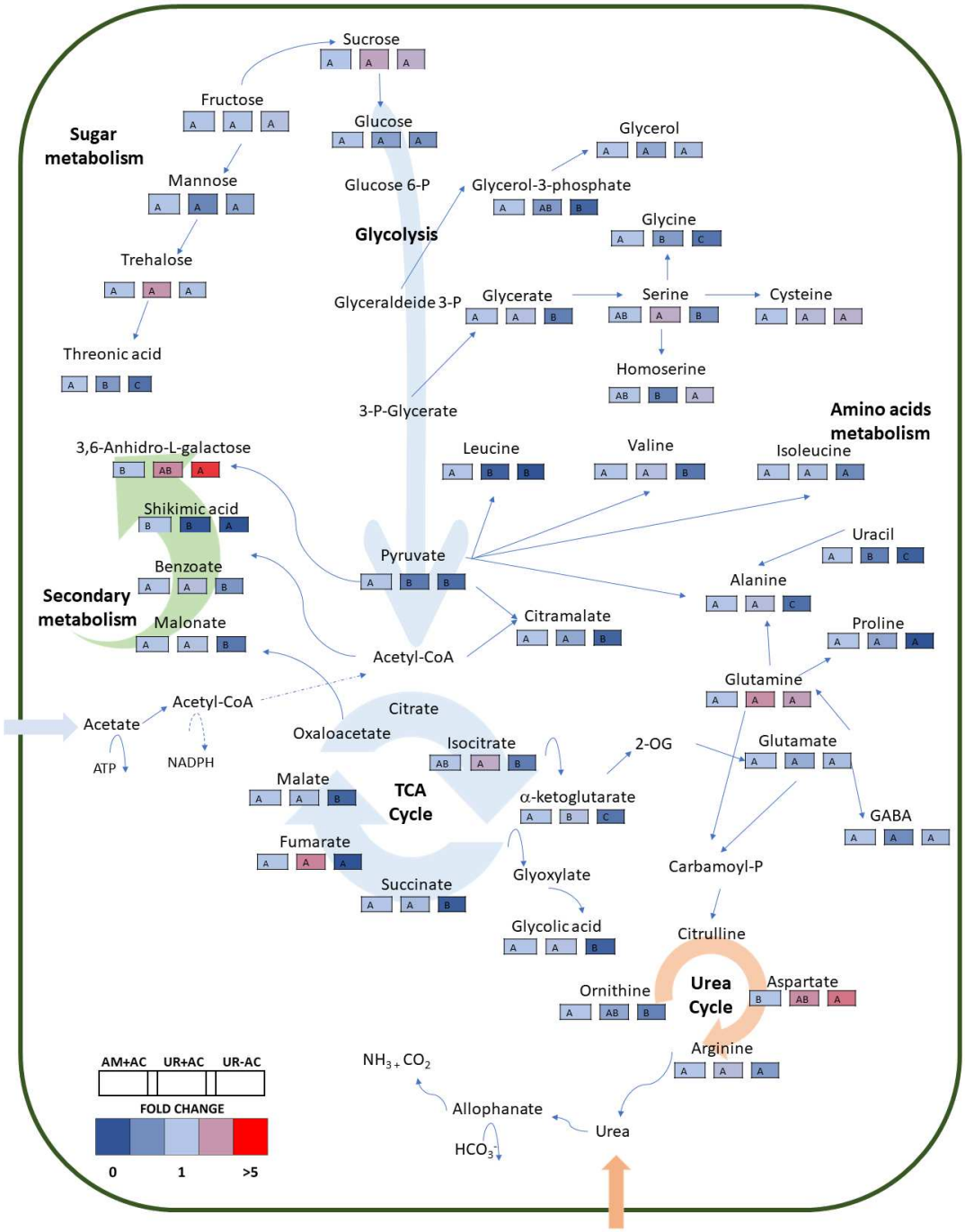


Figure 5 - Metabolite profiling of *C. reinhardtii* cells cultivated under different conditions; TAP with ammonium and acetate (Am+Ac), TAP with urea and acetate (Ur+Ac) and TAP with urea and without acetate (Ur-Ac). Samples were collected in the log phase in 36 h of culture. Heat map represent the changes in relative metabolite contents, determined by proceedings the described in methods. The data were normalized

considering control values of Am+Ac content, the control values was considered as 1 and the other concentrations considered from their concentration. Data were normalized by the internal standard and the dry weight. Means followed by the same letters do not differ by the Tukey test ($P<0.05$).

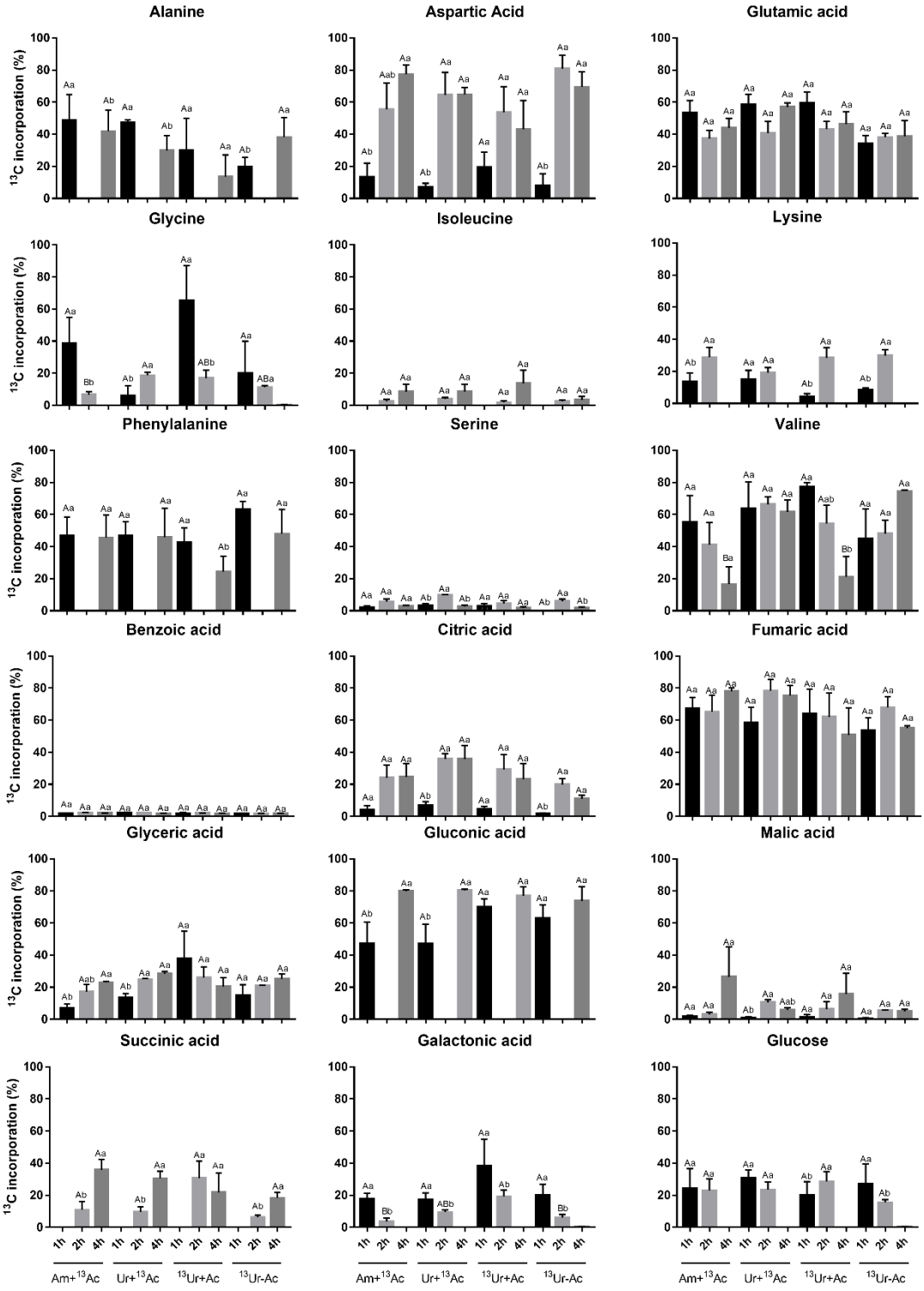


Figure 6 – Percentage of ^{13}C incorporation of metabolites using labelled acetate and urea

in *C. reinhardtii* cells cultivated under different conditions applied for 1, 2 and 4 hours. The ^{13}C labelled substrates were applied in treatments: (i) TAP with ammonium and ^{13}C-Acetate (Am+^{13}Ac), (ii) TAP with urea and ^{13}C-Acetate (Ur+^{13}Ac), (iii) TAP with ^{13}C-Urea and acetate (^{13}Ur+Ac) and (iv) TAP with ^{13}C-Urea without acetate (^{13}Ur-Ac). Means followed by the same letters do not differ by the Tukey test ($P<0.05$), uppercase letters compare the same time in between treatments and lowercase letters compare different times in the same treatment. Treatment conditions are described in Fig. 1 and Material and Methods.

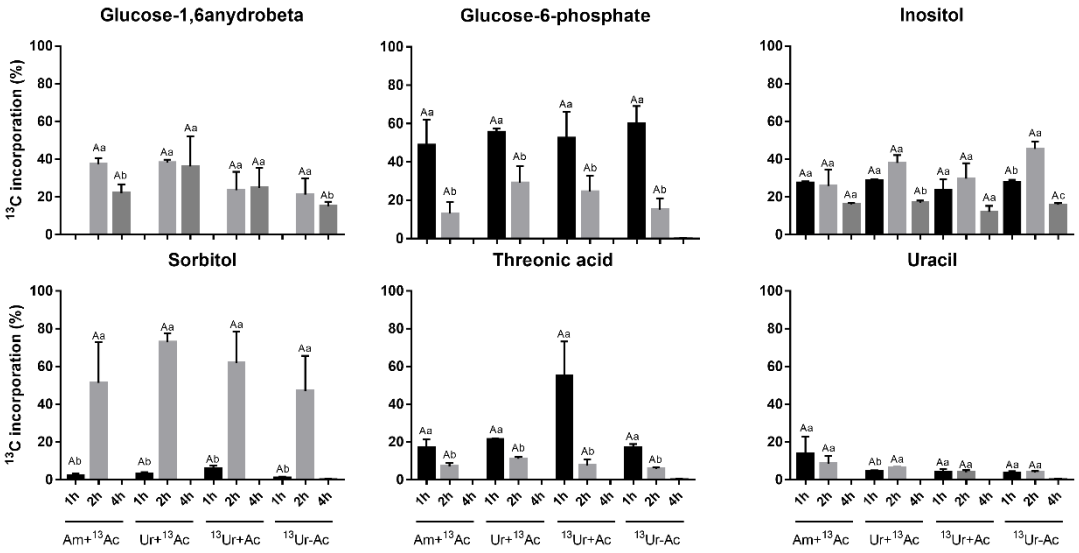


Figure 7 - ^{13}C incorporation of metabolites in percentual during three different times before treatment application. The ^{13}C were applied in four different combinations: TAP with ammonium and ^{13}C acetate (Am+^{13}Ac); TAP with urea and ^{13}C acetate (Ur+^{13}Ac); TAP with ^{13}C Urea and acetate (^{13}Ur+Ac); and TAP with ^{13}C Urea without acetate (^{13}Ur-Ac). Means followed by the same letters do not differ by the Tukey test ($P<0.05$), uppercase letters compare the same time in between treatments and lowercase letters compare different times in the same treatment. Treatment conditions were described in Fig. 1 and Methods.

DISCUSSION

As reported in the literature, the variation in N sources and the nutritional condition affects the growth of microalgae cultures[57,58]. Mixotrophic cultures tend to develop more quickly in a more accelerated process of biomass accumulation, compared to autotrophy. In the present study we confirmed this behaviour, with a greater number of cells being produced in the Am +Ac and Ur +Ac media (Fig. 2A). Conversely, mass per cell was significantly higher for Ur -Ac (Fig. 2B). This variation in growth occurs mainly due to the way in which acetate is metabolized upon entering the cell and how its metabolism affects the other routes of central carbon metabolism, is suggested that acetate modulates changes in routes and cause oxidative stress while it is consumed [59]. A very conclusive parameter in relation to stresses is the pigment content, for which there were no differences between treatments (Fig. 3A-3D)[60]. Combined with this data, a significant decrease was observed in the amino acid content of Am +Ac compared to treatments with urea (Fig. 3F), which made the protein/amino acid ratio to be noticeably greater in Am +Ac (Fig. 3G). Previous studies associate these results with an increase in transcription levels due to the supply of urea, which can affect the cell cycle of *C. reinhardtii* [28,31]. The accumulation of the more abundant C containing compounds in the treatments for the collected phase did not differ for starch and total carbohydrates (Fig. 4). However, it was significantly higher for lipids in Am + Ac. This result is in line with data already described about the tendency to accumulate lipids in mixotrophic condition and the presence of the preferred N source, ammonium [25,61]. The reduced levels of lipids in the mixotrophy condition with urea indicate that the supply of this N source, depending on the cultivation condition, may prioritize other pathways for the use of C [25,35,62].

The results obtained through the metabolite profiling analysis allowed us to investigate more clearly the relationship between the use of urea and the nutritional condition of the

culture (Fig. 5). The variation in nutritional condition and N source did not result in variation in the contents of most sugars such as fructose, glucose and sucrose. The concentration of glycerol-3-phosphate decreases significantly in presence of urea, this metabolite is involved in accelerating the production of triacylglycerols [63], and the modulation of glycerol-3-phosphate oxidase is related to the obtaining of lipids in *C. reinhardtii* [64]. In addition to glycerol-3-phosphate, the TCA cycle intermediates identified were also increased in mixotrophy compared to autotrophy. These results suggest that lipid biosynthesis might stimulated in Am +Ac, although no differences were found in lipids such as methyl palmitate and palmitic acid. Nevertheless, several changes on organic acids and amino acids were observed that play an important role in the C:N ratio. Differences were recorded for serine and metabolites directly involved with its metabolism. Serine metabolism is widely studied in plants for its relationship to photorespiration [65–67]. The increase in serine is a trigger to activate glycerate-serine pathway that is related to regulation of redox balance in stressful conditions[65,68]. Thus, it is suggested that the photorespiratory mechanism is happening in Am +Ac and that in mixotrophy combined with urea supply (Ur +Ac) this activity is attenuated. It is known that the photosynthetic and photorespiratory processes are highly correlated and that the increase in the concentration of acetyl-CoA due to the presence of acetate in mixotrophy stimulates the photorespiratory pathway[66,68]. In this way, changes in the metabolites associated with serine in Ur +Ac tend to be associated with the impact of the presence of urea. The statistically significant decrease in the levels of glycine, which is accumulated from the photorespiratory activity under stress conditions [65], corroborates the hypothesis that the presence of urea mitigates the effect of stress in *C. reinhardtii*. A recent study reveals that, in a continuous light condition, CCM and photorespiration lose their co-regulation, which allows the simultaneous performance of both pathways under the conditions in which the experiment was conducted [69–71]. Interestingly, metabolites

386 that are associated to N stress conditions (like GABA) remained unaltered between
387 treatments [72]. These results indicate that, regardless of the N source offered in this
388 phase, there is no evidence of stress by the differences of N supply. Furthermore, a study
389 of our group where the same treatments were applied concluded that under mixotrophy
390 conditions the organic C supply caused a fast increase of cell number and biomass,
391 saturating the medium during the culture aging and generating impacts on metabolism. In
392 contrast, in autotrophy conditions the culture remained without signs of stress for a long
393 time, keeping the photosynthetic rates and gradual compounds storage during the advance
394 of growth phases. Additionally, was observed that responses of C source from urea
395 become clearer in the acetate absence condition, which permitted suggest that the high C:
396 N ratio in the acetate presence condition masks the effects of the urea supply.

397 The presence of acetate and urea in the culture, as they are organic sources, can both
398 constitute a condition of mixotrophy of the crops. Although the increase in the C: N ratio
399 is much greater for the carbon concentrations added via acetate in the TAP medium, the
400 presence of urea also represents an increase and, consequently, generates changes in the
401 accumulation of cell reserves. The assimilation of acetate in *C. reinhardtii* takes place via
402 the enzyme acetyl-CoA synthetase, which converts the molecule into acetyl-CoA making
403 it available to the glyoxylate and TCA pathways. The decomposition of acetate in the cell,
404 as well as its subsequent reactions, are able to supply all the metabolic substrates of
405 energy metabolism: carbon skeleton, ATP and electrons via reduced coenzymes. As a
406 result of this process, the redox balance is altered in the cell, generating bypasses in the
407 stages of Co2 evolution, inhibiting PSI and changing the pattern of accumulation of
408 reserves, respiration rates and photosynthesis, for example[59,73]. The assimilation of
409 urea does not cause drastic changes in carbon metabolism, however, it is capable of
410 providing additional substrate for the fixation of carbon in inorganic form, since, when
411 assimilated, urea is portioned into components in inorganic sources of C and N. We

suggest that when the two sources are combined: acetate and urea, we can assume that while acetate promotes a rapid displacement of carbon metabolism and forces the activity of photosystems, urea is able to provide additional inorganic carbon to be fixed, mitigating the stressful effects on cell.

The growth and development of organisms is highly dependent on the interaction between C and N [5,74,75]. This interdependence makes the need for C skeletons and N compounds essential in all growth phases, with the use of C and N containing intermediates being widely shared within different pathways. In general, the results of the ^{13}C label incorporation analyses demonstrate that the C from urea can be incorporated in a very similar way of C from acetate for the same metabolites over time (Fig. 6 and Fig. 7). The incorporation of C from acetate does not show significant variation in relation to the available N source. On the other hand, the incorporation of C from urea differ in relation to the condition of autotrophy and mixotrophy for some metabolites. Taken together these results allow us to hypothesise how urea is metabolised under different nutritional conditions (Fig. 6), however further experiments, fluxes and transcriptional analyses are still necessary to. We assume that the carbon from urea tends to, after being fixed, give rise preferentially to fatty acids, while the carbon from acetate is more widely distributed in C and N compounds in the cell and in sugar metabolism. This assumption responds adequately about the use of molecules in the stationary phase, however with the advancement of the phases, cell cycle and metabolization of compounds, what is observed may be very different in phases such as the stationary phase.

CONCLUSIONS

Our study clearly demonstrates that urea is also an important source of C for the cultivation of *C. reinhardtii* and that the nutritional condition is decisive for the metabolic responses after urea supply (Fig. 8). Under mixotrophy conditions, the cells show a high

stimulation of growth depending on the source of organic C (acetate). In the standard condition (Am + Ac), cells tend to replicate quickly, accumulate mass per cell and direct C reserves to the form of lipids. In contrast, with the supply of urea in a condition of mixotrophy (Ur + Ac) the stimulus to the lipids accumulation tends to be attenuated. Under autotrophic conditions in the presence of urea (Ur-Ac) there is a lower rate of replication, less accumulation of mass by cells and the metabolism tends to direct the accumulation of reserves for sugars.

The results suggest that, under the conditions in which the experiment was conducted, the carbonate from urea catabolism in the cell is directed to inorganic C fixation through the Calvin-Benson cycle and not directly in the TCA as with acetate and that the C supply from urea when cultivated with acetate can attenuate the oxidative stresses. This balance into C organic supply with different metabolizations signalized that can be present variations in carbon concentrating mechanisms (CCM) and photorespiration in the culture conditions of experiments. It is also concluded that, regardless of nutritional condition, C from urea catabolism is used in metabolism through the mechanisms of C fixation. Confirmation of these hypotheses will be possible through detailed flow analysis and transcriptional analysis.

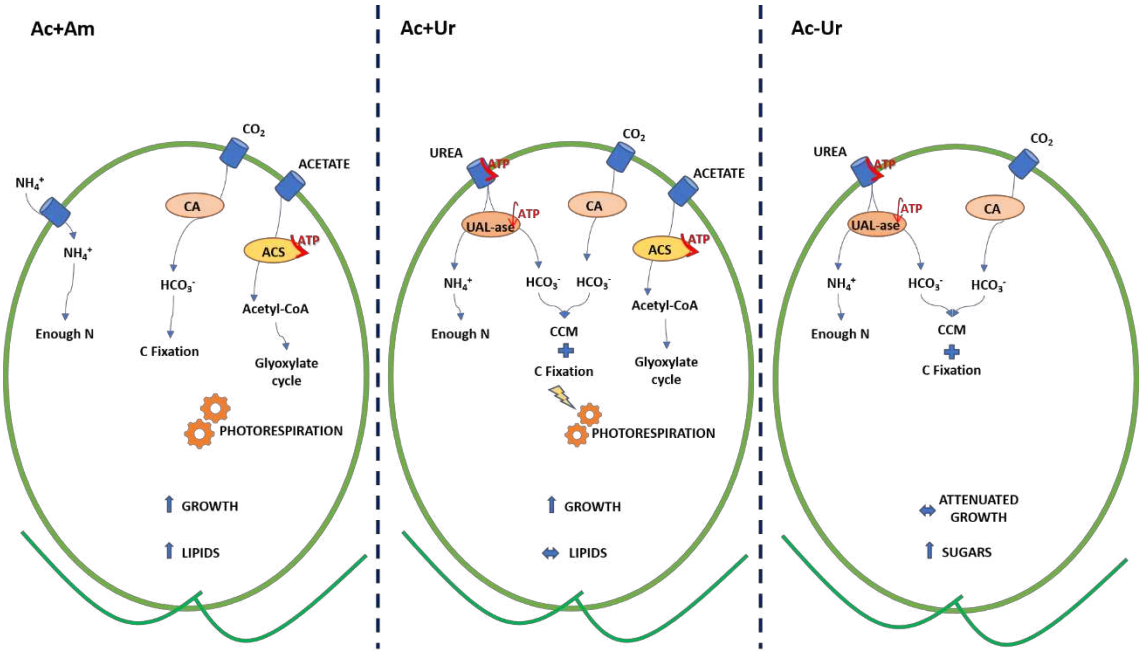


Figure 8 – Summary representation of the impacts of carbon and nitrogen sources in different treatments: Standard TAP medium, with acetate and ammonium, (Ac+Am); TAP medium with acetate and urea as nitrogen source (Ac+ Ur); TAP medium without acetate and with urea as nitrogen source (Ac- Ur). Ellipses represent: CA (carbonic anhydrase), ACS (Acetyl-CoA synthetase), UAL-ase (urea amidolyase). The dimension of the gears is related to the proportion of occurrence of the process. The processes of assimilation and responses of growth and accumulation of compounds are briefly indicated.

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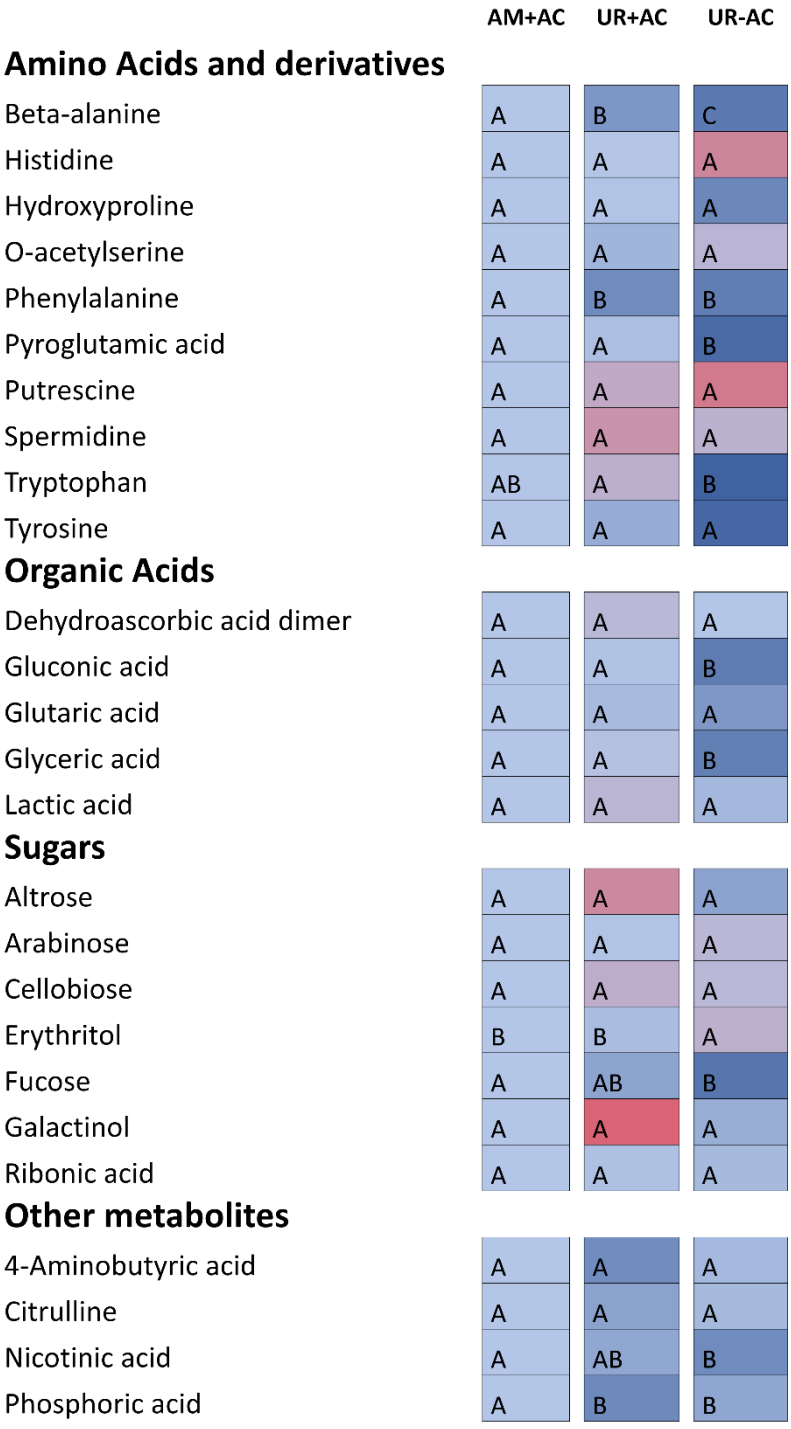
753 **SUPPLEMENTAL FIGURE**



754 Supplemental Figure 1 – Metabolite profiling of *C. reinhardtii* cells cultivated under
755 different conditions; TAP with ammonium and acetate (Am+Ac), TAP with urea and
756 acetate (Ur+Ac) and TAP with urea and without acetate (Ur-Ac). Samples were collected
757 in the log phase in 36 h of culture. Heat map represent the changes in relative metabolite
758 contents, determined by proceedings the described in methods. The data were normalized
759 considering control values of Am+Ac content, the control values was considered as 1 and

760 the other concentrations considered from their concentration. Data were normalized by
761 the internal standard and the dry weight. Means followed by the same letters do not differ
762 by the Tukey test ($P<0.05$).

CAPÍTULO 3- CONCLUSÕES GERAIS

Dada a importância biotecnológica das microalgas os estudos acerca das fontes de elementos essenciais são cada vez mais comuns [1,2]. Compreender de que forma moléculas específicas são aproveitadas por microalgas e os impactos metabólicos do cultivo com esse tipo de suplementação são essenciais para a consolidação de métodos assertivos de produção de compostos de interesse nesses organismos [1,3,4]. Neste contexto, microalga modelo *Chlamydomonas reinhardtii* é bastante importante nos estudos em função da sua facilidade de cultivo e manipulação, amplo referenciamento bibliográfico e por possuir genoma sequenciado e bem anotado [5,6].

As fontes de carbono (C) e nitrogênio (N) são amplamente estudadas por estarem associadas à modulação dos principais produtos dessas microalgas [7]. Dentre as fontes de N, a ureia, uma fonte orgânica desse elemento, é uma das mais corriqueiramente pesquisadas em função da sua ampla disponibilidade e sua utilização comercial e baixo custo [8–10]. Levando-se em conta que trabalhos anteriores já haviam citado que a ureia seria utilizada como fonte de C e N em *C. reinhardtii*[8,11], mas não descreveram de que forma esse processo ocorre, o presente trabalho buscou investigar de que forma essa metabolização de ureia acontece e como esta molécula impacta o metabolismo dessa microalga sob diferentes condições nutricionais. Assim, o presente trabalho representa um importante avanço na compreensão dos mecanismos envolvidos na modulação do metabolismo C:N sob condição de suplementação de ureia neste grupo de organismos.

O primeiro capítulo do trabalho objetivou compreender de que forma o metabolismo de *C. reinhardtii* difere quando cultivado com ureia como fonte de N nas condições nutricionais de mixotrofia e autotrofia. Foram avaliados os impactos temporais da condição nutricional e da fonte de N no metabolismo da microalga. A comparação apenas entre as condições nutricionais confirmou resultados anteriores da literatura que

indicavam um maior crescimento e acúmulo de biomassa em condição mixotrófica comparado à autotrófica [12,13]. Em mixotrofia, o suprimento de C orgânico a partir de acetato acelerou as taxas de crescimento de *C. reinhardtii*, causando rápido aumento do número de células e biomassa, saturando o meio de cultura de forma rápida e gerando impactos no metabolismo. Por outro lado, em condições de autotrofia, a cultura permaneceu sem sinais de estresse por mais tempo, mantendo sua atividade fotossintética e acumulando gradualmente compostos de reserva no decorrer dos estádios de crescimento.

A avaliação mais detalhada dos impactos das diferentes fontes de N e condições nutricionais foi realizada através da mensuração de parâmetros bioquímicos, fisiológicos e de crescimento em dois pontos da fase logarítmica e dois pontos da fase estacionária. A partir desse acompanhamento mais criterioso demonstrou-se que há uma tendência geral de variação temporal dos metabólitos de acordo com a condição nutricional e que a fonte de N tem mais impacto em parâmetros fisiológicos e acúmulo de amido e lipídeos e que esses valores são mais representativos quando considerado todo o período de cultivo. Na condição de mixotrofia o total de clorofilas se manteve estável, as concentrações de compostos tenderam a decrescer temporalmente, e não foram observadas diferenças expressivas no acúmulo de amido e lipídeos de acordo com a fonte de N ofertada. Na condição de autotrofia, por sua vez, observou-se expressivo aumento no total de clorofilas, manutenção das concentrações de proteínas e aminoácidos e decréscimo no total de carboidratos. Adicionalmente, as diferentes fontes de N em autotrofia impactaram o acúmulo de compostos, sendo o acúmulo de amido e lipídeos maior na presença de amônio. Outra diferença relevante foi o aumento da taxa de fotossíntese em condições de autotrofia e de respiração especificamente em condição autotrófica e fornecimento de ureia como fonte nitrogenada. Assim, infere-se que alta relação C: N em condição de mixotrofia inibiria os efeitos do C adicional proveniente da ureia, mas que o carbonato

proveniente do catabolismo de ureia seria aproveitado através de mecanismos concentradores de C impactando em outras respostas no metabolismo da microalga.

De forma a complementar os dados obtidos inicialmente, o segundo capítulo buscou investigar de que forma a ureia é utilizada como fonte de C e N em *C. reinhardtii* e como a presença ou ausência de acetato impacta no aproveitamento desta fonte pelo organismo. Para isto foram analisados dados bioquímicos, metabólicos e incorporação de isótopos de C (^{13}C - Ureia, ^{13}C Acetato), em três tratamentos (TAP + Amônio, TAP + Ureia, TAP, sem acetato + Ureia), na fase logarítmica dos cultivos. Os resultados obtidos confirmaram a incorporação do C proveniente da ureia aos componentes da célula e, adicionalmente, que a utilização de ureia como fonte de N não representa uma situação de estresse por privação de N. O crescimento em condição de mixotrofia é bastante acelerado quando comparado à autotrofia, o que já era descrito anteriormente. Os resultados demonstraram que na fase logarítmica o cultivo com ureia representa acúmulo de lipídeos em mixotrofia e de carboidratos em autotrofia.

Os resultados obtidos a partir da incorporação do isótopo de pesado do C (^{13}C) que o C proveniente da ureia é utilizado pelas células de *C. reinhardtii* e que a condição nutricional interfere nas respostas metabólicas causadas pelo uso da ureia. Em mixotrofia o cultivo tem seu crescimento estimulado pela presença de acetato reduzindo o ciclo celular, acelerando a replicação e aumentando o acúmulo de biomassa. Verificou-se que a fonte de N em condição de mixotrofia afeta o acúmulo de compostos de reserva; enquanto o fornecimento de amônia leva a um maior acúmulo de lipídeos, o uso de ureia tem menor incremento de lipídeos e mantém acúmulo de amido e carboidratos. Por outro lado, em condições de autotrofia, ainda que com o fornecimento de C a partir da ureia, a taxa de replicação e o acúmulo de biomassa por células são menores e o acúmulo de açucares é o principal mecanismo de reservas. Os dados sugerem que o catabolismo da ureia forneça C para o mecanismo concentrador de C (CCM)[14,15] e que a partir daí o

mesmo atua no suprimento de C para a fixação através do ciclo de Calvin-Benson e não diretamente no ciclo TCA, como ocorre com o acetato após metabolização. Esse direcionamento de C para uma via de fixação inorgânica através do CCM seria capaz de atenuar os impactos do rápido crescimento em função do fornecimento de acetato na condição de mixotrofia, o que promoveria estresse oxidativo e atividade fotorrespiratória. Entretanto análises mais específicas em relação ao fluxo metabólico do C, avaliação de parâmetros fisiológicos e fotorrespiração e atividade de enzimas envolvidas na fotorrespiração e estresse oxidativo, nas mesmas condições experimentais conduzidas, são necessárias para que essa hipótese seja comprovada.

Assim, conclui-se que o uso da ureia em *C. reinhardtii* deve ser considerado fonte de N e C nos cultivos e que a alteração da condição nutricional a partir do fornecimento de acetato tem impactos importantes no metabolismo por alterar grandemente a razão C:N. Nas condições em que os experimentos foram conduzidos demonstrou-se que, além da condição nutricional e da fonte de N ofertada, a etapa do crescimento afeta o tipo de metabólito de reserva, bem como o acúmulo dos demais componentes celulares. Esses resultados acerca de padrão de acúmulo de compostos têm impacto direto no planejamento de meios de cultivo com o intuito de estimular a produção de compostos específicos em microalgas, como por exemplo lipídeos e amido, como é o caso de *C. reinhardtii*. A partir dos dados obtidos já é possível selecionar a fonte de N, condição nutricional e fase de crescimento onde os teores de amido, aminoácidos ou clorofila estarão acentuados. Adicionalmente, as inferências feitas em relação ao uso do C proveniente da ureia, embasaram a hipótese de que esta molécula potencialmente estaria envolvida em CCM, diminuição de fotorrespiração em condições de mixotrofia e como atenuadora de estresses oxidativos. Essas novas hipóteses sugeridas pelo trabalho precisam ser avaliadas posteriormente através de análises mais específicas para serem confirmadas. A partir dos dados obtidos já é possível selecionar a fonte de N, condição

nutricional e fase de crescimento onde os teores de amido, aminoácidos ou clorofila estarão acentuados. Além de conclusões relevantes, o trabalho argumenta sobre hipóteses chave que podem servir de base para diversos trabalhos tanto voltados para metabolismo e fisiologia, quanto para produtividade.

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